(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 March 2007 (15.03.2007)

PCT

(10) International Publication Number
WO 2007/030619 A2

(51) International Patent Classification: Not classified

(21) International Application Number: PCT/US2006/034859

(22) International Filing Date: 8 September 2006 (08.09.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

11/223,699	8 September 2005 (08.09.2005)	US
60/727,216	14 October 2005 (14.10.2005)	US
60/733,664	4 November 2005 (04.11.2005)	US

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(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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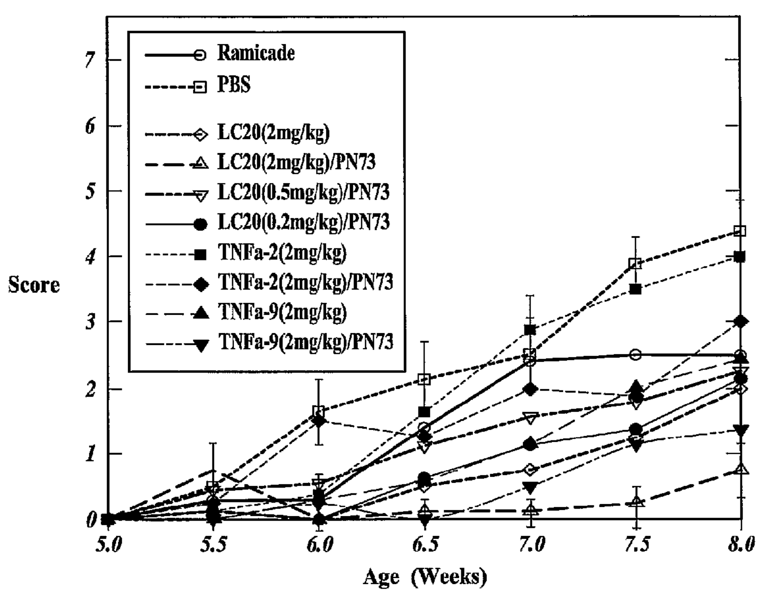
(54) **Title:** PHARMACEUTICAL COMPOSITIONS FOR DELIVERY OF RIBONUCLEIC ACID TO A CELL



(57) **Abstract:** A composition, method for causing uptake in animal cells of double stranded RNA (dsRNA) and reduction of a target mRNA and a use of a mixture for the production of a medicament for the treatment for Tumor Necrosis Factor-alpha (TNF-α) associated inflammatory condition(s) in an animal subject comprising a polynucleotide delivery-enhancing polypeptide and a dsRNA, wherein the polynucleotide delivery-enhancing polypeptide is amphipathic and comprises nucleic acid binding properties are described.

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Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
— *of inventorship (Rule 4.17(iv))*

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

PHARMACEUTICAL COMPOSITIONS FOR DELIVERY OF RIBONUCLEIC ACID TO A CELL

TECHNICAL FIELD

The invention relates to methods and compositions for delivering nucleic acids into cells. More specifically, the invention relates to procedures and preparations for delivering double-stranded polynucleotides into cells to modify expression of target genes to alter a phenotype, such as a disease state or potential, of the cells.

BACKGROUND OF THE INVENTION

Delivering nucleic acids into animal and plant cells has long been an important object of molecular biology research and development. Recent developments in the areas of gene therapy, antisense therapy and RNA interference (RNAi) therapy have created a need to develop more efficient means for introducing nucleic acids into cells.

A diverse array of plasmids and other nucleic acid "vectors" have been developed for delivering large polynucleotide molecules into cells. Typically these vectors incorporate large DNA molecules comprising intact genes for the purpose of transforming target cells to express a gene of scientific or therapeutic interest.

The process by which exogenous nucleic acids are delivered artificially into cells is generally referred to as transfection. Cells can be transfected to uptake a functional nucleic acid from an exogenous source using a variety of techniques and materials. The most commonly used transfection methods are calcium phosphate transfection, and electroporation. A variety of other methods for tranducing cells to deliver exogenous DNA or RNA molecules have been developed, including viral-mediated transduction, cationic lipid or liposomal delivery, and numerous methods that target mechanical or biochemical membrane disruption/penetration (e.g., using detergents, microinjection, or particle guns).

RNA interference is a process of sequence-specific post transcriptional gene silencing in cells initiated by a double-stranded (ds) polynucleotide, usually a dsRNA, that is homologous in sequence to a portion of a targeted messenger RNA (mRNA). Introduction of a suitable dsRNA into cells leads to destruction of endogenous, cognate mRNAs (i.e., mRNAs that share substantial sequence identity with the introduced dsRNA). The dsRNA molecules are cleaved by an RNase III family nuclease called dicer into short-interfering RNAs (siRNAs), which are 19-23 nucleotides (nt) in length. The siRNAs are then incorporated into a multicomponent nuclease complex known as the RNA-induced silencing complex or "RISC." The RISC identifies mRNA

substrates through their homology to the siRNA, and effectuates silencing of gene expression by binding to and destroying the targeted mRNA.

RNA interference is emerging a promising technology for modifying expression of specific genes in plant and animal cells, and is therefore expected to provide useful tools to treat a wide range of diseases and disorders amenable to treatment by modification of endogenous gene expression.

There remains a long-standing need in the art for better tools and methods to deliver siRNAs and other small inhibitory nucleic acids (siNAs) into cells, particularly in view of the fact that existing techniques for delivering nucleic acids to cells are limited by poor efficiency and/or high toxicity of the delivery reagents. Related needs exist for improved methods and formulations to deliver siNAs in an effective amount, in an active and enduring state, and using non-toxic delivery vehicles, to selected cells, tissues, or compartments to mediate regulation of gene expression in a manner that will alter a phenotype or disease state of the targeted cells.

SUMMARY OF THE INVENTION

One aspect of the present invention is a composition comprising a polynucleotide delivery-enhancing polypeptide and a double stranded ribonucleic acid (dsRNA), wherein the polynucleotide delivery-enhancing polypeptide is amphipathic and comprises nucleic acid binding properties. In a related embodiment, the polynucleotide delivery-enhancing polypeptide comprises about 5 to about 40 amino acids, and has all or part of a sequence selected from the group consisting of Poly (Lys, Tryp) 4:1 MW 20,000-50,000, Poly (Orn, Trp) 4:1 20,000-50,000, Mellitin, Histone H1, Histone H3 and Histone H4, SEQ ID NOS 27 to 31, 35 to 42, 45, 47, 50 to 59, 62, 63, 67, 68, 73, 74, 76, 78 to 87, 89 to 92, 94 to 108, 164 to 178 and 180 to 186.

In another embodiment, the composition causes uptake of the dsRNA into an animal cell. In another embodiment, the animal cell is a mammalian cell. In another embodiment, the composition is administered to an animal. In a related embodiment, the animal is a mammal. In another embodiment, the N-terminus of the polynucleotide delivery-enhancing polypeptide is acetylated. In a related embodiment, the N-terminus of the polynucleotide delivery-enhancing polypeptide is pegylated. In yet another embodiment, the dsRNA is a small interfering ribonucleic acid (siRNA) consisting of about 10 to about 40 base pair sequence that is complementary to a portion of a Tumor Necrosis Factor-alpha (TNF-α) gene. In a related embodiment, the dsRNA is a siRNA consisting of about 10 to about 40 base pair sequence selected from the group consisting of SEQ ID NOS 109 to 163 and 187. In another embodiment, the polynucleotide delivery-enhancing polypeptide is admixed, complexed or conjugated to the

dsRNA. In another embodiment, the polynucleotide delivery-enhancing polypeptide binds to the dsRNA. In yet another embodiment, any of the compositions above, further comprising a cationic lipid. In a related embodiment, the cationic lipid is selected from the group consisting of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride, 1,2-bis(oleoyloxy)-3-3-(trimethylammonium)propane, 1,2-dimyristyloxypropyl-3-dimethylhydroxyethylammonium bromide, dimethyldioctadecylammonium bromide, 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminiu m trifluoracetate, 1,3-dioleoyloxy-2-(6-carboxyspermyl)-propylamid, 5-carboxyspermylglycine dioctadecylamide, tetramethyltetrapalmitoyl spermine, tetramethyltetraoleyl spermine, tetramethyltetralauryl spermine, tetramethyltetramyristyl spermine and tetramethyldioleyl spermine, DOTMA (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride), DOTAP (1,2-bis(oleoyloxy)-3,3-(trimethylammonium)propane), DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide), DDAB (dimethyl dioctadecyl ammonium bromide), polyvalent cationic lipids, lipospermines, DOSPA (2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanamini um trifluoro-acetate), DOSPER (1,3-dioleoyloxy-2-(6carboxy spermyl)-propyl-amid, di- and tetra-alkyl-tetra-methyl spermines, TMTPS (tetramethyltetrapalmitoyl spermine), TMTOS (tetramethyltetraoleyl spermine), TMTLS (tetramethlytetralauryl spermine), TMTMS (tetramethyltetramyristyl spermine), TMDOS (tetramethyldioleyl spermine) DOGS (dioctadecyl-amidoglycylspermine (TRANSFECTAM®), cationic lipids combined with non-cationic lipids, DOPE (dioleoylphosphatidylethanolamine), DPhPE (diphytanoylphosphatidylethanolamine) or cholesterol, a cationic lipid composition composed of a 3:1 (w/w) mixture of DOSPA and DOPE, and a 1:1 (w/w) mixture of DOTMA and DOPE.

In another aspect of the present invention is a method for causing uptake of a double stranded ribonucleic acid (dsRNA) into an animal cell, which comprises incubating the animal cells with a mixture comprising a polynucleotide delivery-enhancing polypeptide and the dsRNA, wherein the polynucleotide delivery-enhancing polypeptide is amphipathic and comprises nucleic acid binding properties.

In another aspect of the present invention is a method for modifying expression of a target gene in an animal cell, which comprises incubating the animal cell with a mixture comprising a polynucleotide delivery-enhancing polypeptide, wherein the polynucleotide delivery-enhancing polypeptide is amphipathic and comprises nucleic acid binding properties, and a double stranded ribonucleic acid (dsRNA), wherein the dsRNA is complementary to a region of the target gene.

In a related embodiment, is any of the methods above, the animal cell is a mammalian cell.

In another aspect of the present invention is a method for changing a phenotype of an animal subject, which comprises administering to the animal subject a mixture of a polynucleotide delivery-enhancing polypeptide, wherein the polynucleotide delivery-enhancing polypeptide is amphipathic and comprises nucleic acid binding properties, and a double stranded ribonucleic acid (dsRNA), wherein the dsRNA is complementary to a region of a target gene in the subject. In a related embodiment, the animal may be a mammal.

In another embodiment, is any of the methods above, the polynucleotide delivery-enhancing polypeptide comprises about 5 to about 40 amino acids, and has all or part of a sequence selected from the group consisting of Poly (Lys, Tryp) 4:1 MW 20,000-50,000, Poly (Orn, Trp) 4:1 20,000-50,000, Mellitin, Histone H1, Histone H3 and Histone H4, SEQ ID NOS 27 to 31, 35 to 42, 45, 47, 50 to 59, 62, 63, 67, 68, 73, 74, 76, 78 to 87, 89 to 92, 94 to 108, 164 to 178 and 180 to 186. In a related embodiment, is any of the methods above, the N-terminus of the polynucleotide delivery-enhancing polypeptide is acetylated. In another related embodiment, is any of the methods above, the N-terminus of the polynucleotide delivery-enhancing polypeptide is pegylated. In another embodiment, is any of the methods above, the dsRNA is a small interfering ribonucleic acid (siRNA) consisting of about 10 to about 40 base pair sequence that is complementary to a portion of a Tumor Necrosis Factor-alpha (TNF-α) gene. In a related embodiment, is any of the methods above, the dsRNA is a siRNA consisting of about 10 to about 40 base pair sequence selected from the group consisting of SEQ ID NOS 109 to 163 and 187. In another embodiment, is any of the methods above, the polynucleotide delivery-enhancing polypeptide is admixed, complexed or conjugated to the dsRNA. In yet another embodiment, is any of the methods above, the polynucleotide delivery-enhancing polypeptide binds to the dsRNA. In another embodiment, is any of the methods above, further comprising a cationic lipid. In a related embodiment, is any of the methods above the cationic lipid is selected from the group consisting of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride, 1,2-bis(oleoyloxy)-3-3-(trimethylammonium)propane, 1,2-dimyristyloxypropyl-3-dimethylhydroxyethylammonium bromide, dimethyldioctadecylammonium bromide, 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminiu m trifluoracetate, 1,3-dioleoyloxy-2-(6-carboxyspermyl)-propylamid, 5-carboxyspermylglycine dioctadecylamide, tetramethyltetrapalmitoyl spermine, tetramethyltetraoleyl spermine, tetramethyltetralauryl spermine, tetramethyltetramyristyl spermine and tetramethyldioleyl spermine, DOTMA (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride), DOTAP (1,2-bis(oleoyloxy)-

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In another aspect of the invention is a use of a mixture comprising a polynucleotide delivery-enhancing polypeptide, wherein the polynucleotide delivery-enhancing polypeptide is amphipathic and comprises nucleic acid binding properties, and a double stranded ribonucleic acid (dsRNA) for the production of a medicament for the treatment of a Tumor Necrosis Factor-alpha (TNF-α) associated inflammatory condition(s) in an animal subject, wherein the medicament is capable of reducing TNF-α RNA levels thereby preventing or reducing the occurrence or severity of one or more symptom(s) of the TNF-α associated inflammatory condition(s). In an embodiment, the polynucleotide delivery-enhancing polypeptide comprises about 5 to about 40 amino acids, and has all or part of a sequence selected from the group consisting of Poly (Lys, Tryp) 4:1 MW 20,000-50,000, Poly (Orn, Trp) 4:1 20,000-50,000, Mellitin, Histone H1, Histone H3 and Histone H4, SEQ ID NOS 27 to 31, 35 to 42, 45, 47, 50 to 59, 62, 63, 67, 68, 73, 74, 76, 78 to 87, 89 to 92, 94 to 108, 164 to 178 and 180 to 186. In a related embodiment, the N-terminus of the polynucleotide delivery-enhancing polypeptide is acetylated. In another related embodiment, the N-terminus of the polynucleotide delivery-enhancing polypeptide is pegylated. In another embodiment, the dsRNA is a small interfering ribonucleic acid (siRNA) consisting of about 10 to about 40 base pair sequence that is complementary to a portion of a Tumor Necrosis Factor-alpha (TNF-α) gene. In a related embodiment, the dsRNA is a siRNA consisting of about 10 to about 40 base pair sequence selected from the group consisting of SEQ ID NOS 109 to 163 and 187. In another embodiment, the polynucleotide delivery-enhancing polypeptide is admixed, complexed or conjugated to the dsRNA. In another embodiment, the polynucleotide delivery-enhancing polypeptide binds to the dsRNA. In another embodiment, the animal subject is a mammal.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates peptide-mediated uptake and the effect on cell viability of siRNAs complexed or conjugated to a polynucleotide delivery-enhancing polypeptide of the invention (SEQ ID NO: 35). Cell uptake and cell viability are expressed in percent.

Figure 2 further illustrates peptide-mediated uptake of siRNAs complexed or conjugated to a polynucleotide delivery-enhancing polypeptide of the invention (SEQ ID NO: 35). Cell uptake is expressed as mean fluorescent intensity (MFI).

Figure 3 shows peptide-mediated uptake of siRNAs in human monocytes with several different polynucleotide delivery-enhancing polypeptides.

Figure 4 illustrates the effect on human monocyte viability after exposure to siRNAs complexed with several different polynucleotide delivery-enhancing polypeptides.

Figure 5 shows that siRNA/peptide injected mice have a delayed RA progression comparable to that exhibited by Ramicade-treated subjects. RA progression was measured by a paw scoring index.

Figure 6 provides results of uptake efficacy and viability studies in mouse tail fibroblast cells for PN73 rationally-designed derivative polynucleotide delivery-enhancing polypeptides of the invention.

Figure 7 illustrates that peptide-mediated uptake of siRNAs complexed to a polynucleotide delivery-enhancing polypeptide of the invention does not elicit an interferon response compared to lipofectamine mediated delivery of siRNAs. (A): siRNA complexed with Lipofectamine (B): siRNA complexed with PN73(1:5)

Figure 8 shows that siNAs conjugated to a polynucleotide delivery-enhancing polypeptide have greater knockdown activity *in vitro* than siRNAs complexed with a polynucleotide delivery-enhancing polypeptide.

Figure 9 shows a comparison of cell uptake between cholesterol conjugated siRNAs and unconjugated siNAs with a polynucleotide delivery-enhancing polypeptide.

Figure 10 shows that serum inhibition of cell uptake of cholesterol conjugated siRNAs can be rescued with a polynucleotide delivery-enhancing polypeptide.

DESCRIPTION OF EXEMPLARY EMBODIMENTS OF THE INVENTION

The present invention satisfies these needs and fulfills additional objects and advantages by providing novel compositions and methods that employ a short interfering nucleic acid (siNA), or a precursor thereof, in combination with a polynucleotide delivery-enhancing polypeptide. The polynucleotide delivery-enhancing polypeptide is a natural or artificial

polypeptide selected for its ability to enhance intracellular delivery or uptake of polynucleotides, including siNAs and their precursors.

Within the novel compositions of the invention, the siNA may be admixed or complexed with, or conjugated to, the polynucleotide delivery-enhancing polypeptide to form a composition that enhances intracellular delivery of the siNA as compared to delivery resulting from contacting the target cells with a naked siNA (i.e., siNA without the delivery-enhancing polypeptide present).

In certain embodiments of the invention, the polynucleotide delivery-enhancing polypeptide is a histone protein, or a polypeptide or peptide fragment, derivative, analog, or conjugate thereof. Within these embodiments, the siNA is admixed, complexed or conjugated with one or more full length histone proteins or polypeptides corresponding at least in part to a partial sequence of a histone protein, for example of one or more of the following histones: histone H1, histone H2A, histone H2B, histone H3 or histone H4, or one or more polypeptide fragments or derivatives thereof comprising at least a partial sequence of a histone protein, typically at least 5-10 or 10-20 contiguous residues of a native histone protein. In more detailed embodiments, the siRNA/histone mixture, complex or conjugate is substantially free of amphipathic compounds. In other detailed embodiments, the siNA that is admixed, complexed, or conjugated with the histone protein or polypeptide will comprise a double-stranded double-stranded RNA, for example a double-stranded RNA that has 30 or fewer nucleotides, and is a short interfering RNA (siRNA). In exemplary embodiments, the histone polynucleotide delivery-enhancing polypeptide comprises a fragment of histone H2B, as exemplified by the polynucleotide delivery-enhancing polypeptide designated PN73 described herein below. In yet additional detailed embodiments, the polynucleotide delivery-enhancing polypeptide may be pegylated to improve stability and/or efficacy, particularly in the context of in vivo administration.

Within additional embodiments of the invention, the polynucleotide delivery-enhancing polypeptide is selected or rationally designed to comprise an amphipathic amino acid sequence. For example, useful polynucleotide delivery-enhancing polypeptides may be selected which comprise a plurality of non-polar or hydrophobic amino acid residues that form a hydrophobic sequence domain or motif, linked to a plurality of charged amino acid residues that form a charged sequence domain or motif, yielding an amphipathic peptide.

In other embodiments, the polynucleotide delivery-enhancing polypeptide is selected to comprise a protein transduction domain or motif, and a fusogenic peptide domain or motif. A protein transduction domain is a peptide sequence that is able to insert into and preferably transit

through the membrane of cells. A fusogenic peptide is a peptide that destabilizes a lipid membrane, for example a plasma membrane or membrane surrounding an endosome, which may be enhanced at low pH. Exemplary fusogenic domains or motifs are found in a broad diversity of viral fusion proteins and in other proteins, for example fibroblast growth factor 4 (FGF4).

To rationally design polynucleotide delivery-enhancing polypeptides of the invention, a protein transduction domain is employed as a motif that will facilitate entry of the nucleic acid into a cell through the plasma membrane. In certain embodiments, the transported nucleic acid will be encapsulated in an endosome. The interior of endosomes has a low pH resulting in the fusogenic peptide motif destabilizing the membrane of the endosome. The destabilization and breakdown of the endosome membrane allows for the release of the siNA into the cytoplasm where the siNA can associate with a RISC complex and be directed to its target mRNA.

Examples of protein transduction domains for optional incorporation into polynucleotide delivery-enhancing polypeptides of the invention include:

1. TAT protein transduction domain (PTD) (SEQ ID NO: 1) KRRQRRR;
2. Penetratin PTD (SEQ ID NO: 2) RQIKIWFQNRRMKWKK;
3. VP22 PTD (SEQ ID NO: 3) DAATATRGRSAASRPTERPRAPARSASRPRRPVD;
4. Kaposi FGF signal sequences (SEQ ID NO: 4) AAVALLPAVLLALLAP, and SEQ ID NO: 5) AAVLLPVLLPVLLAAP;
5. Human β3 integrin signal sequence (SEQ ID NO: 6) VTVLALGALAGVGVG;
6. gp41 fusion sequence (SEQ ID NO: 7) GALFLGWLGAAGSTMGA;
7. *Caiman crocodylus* Ig(v) light chain (SEQ ID NO: 8) MGLGLHLLVLAAALQGA;
8. hCT-derived peptide (SEQ ID NO: 9) LGTYTQDFNKFHTFPQTAIGVGAP;
9. Transportan (SEQ ID NO: 10) GWTLNSAGYLLKINLKALAALAKKIL;
10. Loligomer (SEQ ID NO: 11) TPPKKKRKVEDPKKKK;
11. Arginine peptide (SEQ ID NO: 12) RRRRRRR; and
12. Amphiphilic model peptide (SEQ ID NO: 13) KLALKLALKALKAALKLA.

Examples of viral fusion peptides fusogenic domains for optional incorporation into polynucleotide delivery-enhancing polypeptides of the invention include:

1. Influenza HA2 (SEQ ID NO: 14) GLFGAIAGFIENGWEG;
2. Sendai F1 (SEQ ID NO: 15) FFGAVIGTIALGVATA;
3. Respiratory Syncytial virus F1 (SEQ ID NO: 16) FLGFLLGVGSAIASGV;
4. HIV gp41 (SEQ ID NO: 17) GVFVLGFLGFLATAGS; and
5. Ebola GP2 (SEQ ID NO: 18) GAAIGLAWIPYFGPAA.

Within yet additional embodiments of the invention, polynucleotide delivery-enhancing polypeptides are provided that incorporate a DNA-binding domain or motif which facilitates polypeptide-siNA complex formation and/or enhances delivery of siNAs within the methods and compositions of the invention. Exemplary DNA binding domains in this context include various "zinc finger" domains as described for DNA-binding regulatory proteins and other proteins identified in Table 1, below (see, e.g., Simpson, et al., *J. Biol. Chem. 278*:28011-28018, 2003).

Table 1:

Exemplary Zinc Finger Motifs of Different DNA-Binding Proteins

C_2H_2 Zinc finger motif

	665	675	685	695	705	715
Sp1	ACTCPYCKDS	EGRGSG----	DPGKKKQHIC	HIQGCGKVYG	KTSHLRAHLR	WHTGERPFMC
Sp2	ACTCPNCKDG	EKRS------	GEQGKKKHVC	HIPDCGKTFR	KTSLLRAHVR	LHTGERPFVC
Sp3	ACTCPNCKEG	GGRGTN----	-LGKKKQHIC	HIPGCGKVYG	KTSHLRAHLR	WHSGERPFVC
Sp4	ACSCPNCREG	EGRGSN----	EPGKKKQHIC	HIEGCGKVYG	KTSHLRAHLR	WHTGERPFIC
DrosBtd	RCTCPNCTNE	MSGLPPIVGP	DERGRKQHIC	HIPGCERLYG	KASHLKTHLR	WHTGERPFLC
DrosSp	TCDCPNCQEA	ERLGPAGV--	HLRKKNIHSC	HIPGCGKVYG	KTSHLKAHLR	WHTGERPFVC
CeT22C8.5	RCTCPNCKAI	KHG-------	DRGSQHTHLC	SVPGCGKTYK	KTSHLRAHLR	KHTGDRPFVC
Y40B1A.4	PQISLKKKIF	FFIFSNFR--	GDGKSRIHIC	HL--CNKTYG	KTSHLRAHLR	GHAGNKPFAC

Prosite pattern

C-x(2,4)-C-x(12)-H-x(3)-H

*The table demonstrates a conservative zinc fingerer motif for double strand DNA binding which is characterized by the C-x(2,4)-C-x(12)-H-x(3)-H motif pattern (SEQ ID NO: 188), which itself can be used to select and design additional polynucleotide delivery-enhancing polypeptides according to the invention.

**The sequences shown in Table 1, for Sp1, Sp2, Sp3, Sp4, DrosBtd, DrosSp, CeT22C8.5, and Y4pB1A.4, are herein assigned SEQ ID NO:s 19, 20, 21, 22, 23, 24, 25, and 26, respectively.

Alternative DNA binding domains useful for constructing polynucleotide delivery-enhancing polypeptides of the invention include, for example, portions of the HIV Tat protein sequence (see, Examples, below).

Within exemplary embodiments of the invention described herein below, polynucleotide delivery-enhancing polypeptides may be rationally designed and constructed by combining any of the foregoing structural elements, domains or motifs into a single polypeptide effective to mediate enhanced delivery of siNAs into target cells. For example, a protein transduction domain of the TAT polypeptide was fused to the N-terminal 20 amino acids of the influenza

virus hemagglutinin protein, termed HA2, to yield one exemplary polynucleotide delivery-enhancing polypeptide herein. Various other polynucleotide delivery-enhancing polypeptide constructs are provided in the instant disclosure, evincing that the concepts of the invention are broadly applicable to create and use a diverse assemblage of effective polynucleotide delivery-enhancing polypeptides for enhancing siNA delivery.

Yet additional exemplary polynucleotide delivery-enhancing polypeptides within the invention may be selected from the following peptides: WWETWKPFQCRICMRNFSTRQARRNHRRRHR (SEQ ID NO: 27); GKINLKALAALAKKIL (SEQ ID NO: 28), RVIRVWFQNKRCKDKK (SEQ ID NO: 29), GRKKRRQRRRPPQGRKKRRQRRRPPQGRKKRRQRRRPPQ (SEQ ID NO: 30), GEQIAQLIAGYIDIILKKKKSK (SEQ ID NO: 31), Poly Lys-Trp, 4:1, MW 20,000-50,000; and Poly Orn-Trp, 4:1, MW 20,000-50,000. Additional polynucleotide delivery-enhancing polypeptides that are useful within the compositions and methods herein comprise all or part of the mellitin protein sequence.

Still other exemplary polynucleotide delivery-enhancing polypeptides are identified in the examples below. Any one or combination of these peptides may be selected or combined to yield effective polynucleotide delivery-enhancing polypeptide reagents to induce or facilitate intracellular delivery of siNAs within the methods and compositions of the invention.

In more detailed aspects of the invention, the mixture, complex or conjugate comprising a siRNA and a polynucleotide delivery-enhancing polypeptide can be optionally combined with (e.g., admixed or complexed with) a cationic lipid, such as LIPOFECTIN®. In this context it is unexpectedly disclosed herein that polynucleotide delivery-enhancing polypeptides complexed or conjugated to a siRNA alone will effectuate delivery of the siNA sufficient to mediate gene silencing by RNAi. However, it is further unexpectedly disclosed herein that a siRNA/polynucleotide delivery-enhancing polypeptide complex or conjugate will exhibit even greater activity for mediating siNA delivery and gene silencing when admixed or complexed with a cationic lipid, such as lipofectin. To produce these compositions comprised of a polynucleotide delivery-enhancing polypeptide, siRNA and a cationic lipid, the siRNA and peptide may be mixed together first in a suitable medium such as a cell culture medium, after which the cationic lipid is added to the mixture to form a siRNA/delivery peptide/cationic lipid composition. Optionally, the peptide and cationic lipid can be mixed together first in a suitable medium such as a cell culture medium, whereafter the siRNA can be added to form the siRNA/delivery peptide/cationic lipid composition.

Examples of useful cationic lipids within these aspects of the invention include N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride, 1,2-bis(oleoyloxy)-3-3-(trimethylammonium)propane, 1,2-dimyristyloxypropyl-3-dimethylhydroxyethylammonium bromide, and dimethyldioctadecylammonium bromide, 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminiu m trifluoracetate, 1,3-dioleoyloxy-2-(6-carboxyspermyl)-propylamid, 5-carboxyspermylglycine dioctadecylamide, tetramethyltetrapalmitoyl spermine, tetramethyltetraoleyl spermine, tetramethyltetralauryl spermine, tetramethyltetramyristyl spermine and tetramethyldioleyl spermine. DOTMA (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride), DOTAP (1,2-bis(oleoyloxy)-3,3-(trimethylammonium)propane), DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide) or DDAB (dimethyl dioctadecyl ammonium bromide). Polyvalent cationic lipids include lipospermines, specifically DOSPA (2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanamini um trifluoro-acetate) and DOSPER (1,3-dioleoyloxy-2-(6carboxy spermyl)-propyl-amid, and the di- and tetra-alkyl-tetra-methyl spermines, including but not limited to TMTPS (tetramethyltetrapalmitoyl spermine), TMTOS (tetramethyltetraoleyl spermine), TMTLS (tetramethlytetralauryl spermine), TMTMS (tetramethyltetramyristyl spermine) and TMDOS (tetramethyldioleyl spermine) DOGS (dioctadecyl-amidoglycylspermine (TRANSFECTAM®). Other useful cationic lipids are described, for example, in U.S. Patent No. 6,733,777; U.S. Patent No. 6,376,248; U.S. Patent No. 5,736,392; U.S. Patent No. 5,686,958; U.S. Patent No. 5,334,761 and U.S. Patent No. 5,459,127.

Cationic lipids are optionally combined with non-cationic lipids, particularly neutral lipids, for example lipids such as DOPE (dioleoylphosphatidylethanolamine), DPhPE (diphytanoylphosphatidylethanolamine) or cholesterol. A cationic lipid composition composed of a 3:1 (w/w) mixture of DOSPA and DOPE or a 1:1 (w/w) mixture of DOTMA and DOPE (LIPOFECTIN®, Invitrogen) are generally useful in transfecting compositions of this invention. Preferred transfection compositions are those which induce substantial transfection of a higher eukaryotic cell line.

In exemplary embodiments, the instant invention features compositions comprising a small nucleic acid molecule, such as short interfering nucleic acid (siNA), a short interfering RNA (siRNA), a double-stranded RNA (dsRNA), micro-RNA (mRNA), or a short hairpin RNA (shRNA), admixed or complexed with, or conjugated to, a polynucleotide delivery-enhancing polypeptide.

As used herein, the term "short interfering nucleic acid", "siNA", "short interfering RNA", "siRNA", "short interfering nucleic acid molecule", "short interfering oligonucleotide molecule", or "chemically-modified short interfering nucleic acid molecule", refers to any nucleic acid molecule capable of inhibiting or down regulating gene expression or viral replication, for example by mediating RNA interference "RNAi" or gene silencing in a sequence-specific manner. Within exemplary embodiments, the siNA is a double-stranded polynucleotide molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence in a target nucleic acid molecule for down regulating expression, or a portion thereof, and the sense region comprises a nucleotide sequence corresponding to (i.e., which is substantially identical in sequence to) the target nucleic acid sequence or portion thereof.

"siNA" means a small interfering nucleic acid, for example a siRNA, that is a short-length double-stranded nucleic acid (or optionally a longer precursor thereof), and which is not unacceptably toxic in target cells. The length of useful siNAs within the invention will in certain embodiments be optimized at a length of approximately 21 to 23 bp long. However, there is no particular limitation in the length of useful siNAs, including siRNAs. For example, siNAs can initially be presented to cells in a precursor form that is substantially different than a final or processed form of the siNA that will exist and exert gene silencing activity upon delivery, or after delivery, to the target cell. Precursor forms of siNAs may, for example, include precursor sequence elements that are processed, degraded, altered, or cleaved at or following the time of delivery to yield a siNA that is active within the cell to mediate gene silencing. Thus, in certain embodiments, useful siNAs within the invention will have a precursor length, for example, of approximately 100-200 base pairs, 50-100 base pairs, or less than about 50 base pairs, which will yield an active, processed siNA within the target cell. In other embodiments, a useful siNA or siNA precursor will be approximately10 to 49 bp, 15 to 35 bp, or about 21 to 30 bp in length.

In certain embodiments of the invention, as noted above, polynucleotide delivery-enhancing polypeptides are used to facilitate delivery of larger nucleic acid molecules than conventional siNAs, including large nucleic acid precursors of siNAs. For example, the methods and compositions herein may be employed for enhancing delivery of larger nucleic acids that represent "precursors" to desired siNAs, wherein the precursor amino acids may be cleaved or otherwise processed before, during or after delivery to a target cell to form an active siNA for modulating gene expression within the target cell. For example, a siNA precursor polynucleotide may be selected as a circular, single-stranded polynucleotide, having two or more

loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence in a target nucleic acid molecule or a portion thereof, and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof, and wherein the circular polynucleotide can be processed either in vivo or in vitro to generate an active siNA molecule capable of mediating RNAi.

In mammalian cells, dsRNAs longer than 30 base pairs can activate the dsRNA-dependent kinase PKR and 2'-5'-oligoadenylate synthetase, normally induced by interferon. The activated PKR inhibits general translation by phosphorylation of the translation factor eukaryotic initiation factor 2α (eIF2α), while 2'-5'-oligoadenylate synthetase causes nonspecific mRNA degradation via activation of RNase L. By virtue of their small size (referring particularly to non-precursor forms), usually less than 30 base pairs, and most commonly between about 17-19, 19-21, or 21-23 base pairs, the siNAs of the present invention avoid activation of the interferon response.

In contrast to the nonspecific effect of long dsRNA, siRNA can mediate selective gene silencing in the mammalian system. Hairpin RNAs, with a short loop and 19 to 27 base pairs in the stem, also selectively silence expression of genes that are homologous to the sequence in the double-stranded stem. Mammalian cells can convert short hairpin RNA into siRNA to mediate selective gene silencing.

RISC mediates cleavage of single stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex. Studies have shown that 21 nucleotide siRNA duplexes are most active when containing two nucleotide 3'-overhangs. Furthermore, complete substitution of one or both siRNA strands with 2'-deoxy (2'-H) or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of the 3'-terminal siRNA overhang nucleotides with deoxy nucleotides (2'-H) has been reported to be tolerated.

Studies have shown that replacing the 3'-overhanging segments of a 21-mer siRNA duplex having 2 nucleotide 3' overhangs with deoxyribonucleotides does not have an adverse effect on RNAi activity. Replacing up to 4 nucleotides on each end of the siRNA with deoxyribonucleotides has been reported to be well tolerated whereas complete substitution with deoxyribonucleotides results in no RNAi activity.

Alternatively, the siNAs can be delivered as single or multiple transcription products expressed by a polynucleotide vector encoding the single or multiple siNAs and directing their expression within target cells. In these embodiments the double-stranded portion of a final

transcription product of the siRNAs to be expressed within the target cell can be, for example, 15 to 49 bp, 15 to 35 bp, or about 21 to 30 bp long. Within exemplary embodiments, double-stranded portions of siNAs, in which two strands pair up, are not limited to completely paired nucleotide segments, and may contain nonpairing portions due to mismatch (the corresponding nucleotides are not complementary), bulge (lacking in the corresponding complementary nucleotide on one strand), overhang, and the like. Nonpairing portions can be contained to the extent that they do not interfere with siNA formation. In more detailed embodiments, a "bulge" may comprise 1 to 2 non-pairing nucleotides, and the double-stranded region of siNAs in which two strands pair up may contain from about 1 to 7, or about 1 to 5 bulges. In addition, "mismatch" portions contained in the double-stranded region of siNAs may be present in numbers from about 1 to 7, or about 1 to 5. Most often in the case of mismatches, one of the nucleotides is guanine, and the other is uracil. Such mismatching may be attributable, for example, to a mutation from C to T, G to A, or mixtures thereof, in a corresponding DNA coding for sense RNA, but other cause are also contemplated. Furthermore, in the present invention the double-stranded region of siNAs in which two strands pair up may contain both bulge and mismatched portions in the approximate numerical ranges specified.

The terminal structure of siNAs of the invention may be either blunt or cohesive (overhanging) as long as the siNA retains its activity to silence expression of target genes. The cohesive (overhanging) end structure is not limited only to the 3' overhang as reported by others. On the contrary, the 5' overhanging structure may be included as long as it is capable of inducing a gene silencing effect such as by RNAi. In addition, the number of overhanging nucleotides is not limited to reported limits of 2 or 3 nucleotides, but can be any number as long as the overhang does not impair gene silencing activity of the siNA. For example, overhangs may comprise from about 1 to 8 nucleotides, more often from about 2 to 4 nucleotides. The total length of siNAs having cohesive end structure is expressed as the sum of the length of the paired double-stranded portion and that of a pair comprising overhanging single-strands at both ends. For example, in the exemplary case of a 19 bp double-stranded RNA with 4 nucleotide overhangs at both ends, the total length is expressed as 23 bp. Furthermore, since the overhanging sequence may have low specificity to a target gene, it is not necessarily complementary (antisense) or identical (sense) to the target gene sequence. Furthermore, as long as the siNA is able to maintain its gene silencing effect on the target gene, it may contain low molecular weight structure (for example a natural RNA molecule such as tRNA, rRNA or viral RNA, or an artificial RNA molecule), for example, in the overhanging portion at one end.

In addition, the terminal structure of the siNAs may have a stem-loop structure in which ends of one side of the double-stranded nucleic acid are connected by a linker nucleic acid, e.g., a linker RNA. The length of the double-stranded region (stem-loop portion) can be, for example, 15 to 49 bp, often 15 to 35 bp, and more commonly about 21 to 30 bp long. Alternatively, the length of the double-stranded region that is a final transcription product of siNAs to be expressed in a target cell may be, for example, approximately 15 to 49 bp, 15 to 35 bp, or about 21 to 30 bp long. When linker segments are employed, there is no particular limitation in the length of the linker as long as it does not hinder pairing of the stem portion. For example, for stable pairing of the stem portion and suppression of recombination between DNAs coding for this portion, the linker portion may have a clover-leaf tRNA structure. Even if the linker has a length that would hinder pairing of the stem portion, it is possible, for example, to construct the linker portion to include introns so that the introns are excised during processing of a precursor RNA into mature RNA, thereby allowing pairing of the stem portion. In the case of a stem-loop siRNA, either end (head or tail) of RNA with no loop structure may have a low molecular weight RNA. As described above, these low molecular weight RNAs may include a natural RNA molecule, such as tRNA, rRNA or viral RNA, or an artificial RNA molecule.

The siNA can also comprise a single stranded polynucleotide having nucleotide sequence complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof (for example, where such siNA molecule does not require the presence within the siNA molecule of nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof), wherein the single stranded polynucleotide can further comprise a terminal phosphate group, such as a 5'-phosphate (see for example, Martinez, et al., *Cell. 110*:563-574, 2002, and Schwarz, et al., *Molecular Cell 10*:537-568, 2002, or 5',3'-diphosphate.

As used herein, the term siNA molecule is not limited to molecules containing only naturally-occurring RNA or DNA, but also encompasses chemically-modified nucleotides and non-nucleotides. In certain embodiments, the short interfering nucleic acid molecules of the invention lack 2'-hydroxy (2'-OH) containing nucleotides. In certain embodiments short interfering nucleic acids do not require the presence of nucleotides having a 2'-hydroxy group for mediating RNAi and as such, short interfering nucleic acid molecules of the invention optionally do not include any ribonucleotides (e.g., nucleotides having a 2'-OH group). Such siNA molecules that do not require the presence of ribonucleotides within the siNA molecule to support RNAi can however have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. Optionally,

siNA molecules can comprise ribonucleotides at about 5, 10, 20, 30, 40, or 50% of the nucleotide positions.

As used herein, the term siNA is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi, for example short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (mRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering modified oligonucleotide, chemically-modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), and others.

In other embodiments, siNA molecules for use within the invention may comprise separate sense and antisense sequences or regions, wherein the sense and antisense regions are covalently linked by nucleotide or non-nucleotide linker molecules, or are alternately non-covalently linked by ionic interactions, hydrogen bonding, van der waals interactions, hydrophobic interactions, and/or stacking interactions.

"Antisense RNA" is an RNA strand having a sequence complementary to a target gene mRNA, and thought to induce RNAi by binding to the target gene mRNA. "Sense RNA" has a sequence complementary to the antisense RNA, and annealed to its complementary antisense RNA to form siRNA. These antisense and sense RNAs have been conventionally synthesized with an RNA synthesizer.

As used herein, the term "RNAi construct" is a generic term used throughout the specification to include small interfering RNAs (siRNAs), hairpin RNAs, and other RNA species which can be cleaved in vivo to form siRNAs. RNAi constructs herein also include expression vectors (also referred to as RNAi expression vectors) capable of giving rise to transcripts which form dsRNAs or hairpin RNAs in cells, and/or transcripts which can produce siRNAs in vivo. Optionally, the siRNA include single strands or double strands of siRNA.

An siHybrid molecule is a double-stranded nucleic acid that has a similar function to siRNA. Instead of a double-stranded RNA molecule, an siHybrid is comprised of an RNA strand and a DNA strand. Preferably, the RNA strand is the antisense strand as that is the strand that binds to the target mRNA. The siHybrid created by the hybridization of the DNA and RNA strands have a hybridized complementary portion and preferably at least one 3'overhanging end.

siNAs for use within the invention can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (i.e., each strand comprises nucleotide sequence that is complementary to nucleotide sequence in the other strand; such as where the antisense strand and sense strand form a duplex or double stranded structure, for example wherein the double

stranded region is about 19 base pairs). The antisense strand may comprise a nucleotide sequence that is complementary to a nucleotide sequence in a target nucleic acid molecule or a portion thereof, and the sense strand may comprise a nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. Alternatively, the siNA can be assembled from a single oligonucleotide, where the self-complementary sense and antisense regions of the siNA are linked by means of a nucleic acid-based or non-nucleic acid-based linker(s).

Within additional embodiments, siNAs for intracellular delivery according to the methods and compositions of the invention can be a polynucleotide with a duplex, asymmetric duplex, hairpin or asymmetric hairpin secondary structure, having self-complementary sense and antisense regions, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence in a separate target nucleic acid molecule or a portion thereof, and the sense region comprises a nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof.

Non-limiting examples of chemical modifications that can be made in an siNA include without limitation phosphorothioate internucleotide linkages, 2'-deoxyribonucleotides, 2'-O-methyl ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, "universal base" nucleotides, "acyclic" nucleotides, 5-C-methyl nucleotides, and terminal glyceryl and/or inverted deoxy abasic residue incorporation. These chemical modifications, when used in various siNA constructs, are shown to preserve RNAi activity in cells while at the same time, dramatically increasing the serum stability of these compounds.

In a non-limiting example, the introduction of chemically-modified nucleotides into nucleic acid molecules provides a powerful tool in overcoming potential limitations of in vivo stability and bioavailability inherent to native RNA molecules that are delivered exogenously. For example, the use of chemically-modified nucleic acid molecules can enable a lower dose of a particular nucleic acid molecule for a given therapeutic effect since chemically-modified nucleic acid molecules tend to have a longer half-life in serum. Furthermore, certain chemical modifications can improve the bioavailability of nucleic acid molecules by targeting particular cells or tissues and/or improving cellular uptake of the nucleic acid molecule. Therefore, even if the activity of a chemically-modified nucleic acid molecule is reduced as compared to a native nucleic acid molecule, for example, when compared to an all-RNA nucleic acid molecule, the overall activity of the modified nucleic acid molecule can be greater than that of the native molecule due to improved stability and/or delivery of the molecule. Unlike native unmodified siNA, chemically-modified siNA can also minimize the possibility of activating interferon activity in humans.

The siNA molecules described herein, the antisense region of a siNA molecule of the invention can comprise a phosphorothioate internucleotide linkage at the 3'-end of said antisense region. In any of the embodiments of siNA molecules described herein, the antisense region can comprise about one to about five phosphorothioate internucleotide linkages at the 5'-end of said antisense region. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs of a siNA molecule of the invention can comprise ribonucleotides or deoxyribonucleotides that are chemically-modified at a nucleic acid sugar, base, or backbone. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs can comprise one or more universal base ribonucleotides. In any of the embodiments of siNA molecules described herein, the 3'-terminal nucleotide overhangs can comprise one or more acyclic nucleotides.

For example, in a non-limiting example, the invention features a chemically-modified short interfering nucleic acid (siNA) having about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in one siNA strand. In yet another embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) individually having about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in both siNA strands. The phosphorothioate internucleotide linkages can be present in one or both oligonucleotide strands of the siNA duplex, for example in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more phosphorothioate internucleotide linkages at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (e.g., about 1, 2, 3, 4, 5, or more) consecutive phosphorothioate internucleotide linkages at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) pyrimidine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands. In yet another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) purine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands.

An siNA molecule may be comprised of a circular nucleic acid molecule, wherein the siNA is about 38 to about 70 (e.g., about 38, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 18 to about 23 (e.g., about 18, 19, 20, 21, 22, or 23) base pairs wherein the circular oligonucleotide forms a dumbbell shaped structure having about 19 base pairs and 2 loops.

A circular siNA molecule contains two loop motifs, wherein one or both loop portions of the siNA molecule is biodegradable. For example, a circular siNA molecule of the invention is designed such that degradation of the loop portions of the siNA molecule in vivo can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

Modified nucleotides present in siNA molecules, preferably in the antisense strand of the siNA molecules, but also optionally in the sense and/or both antisense and sense strands, comprise modified nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example, Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag ed., 1984). As such, chemically modified nucleotides present in the siNA molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also optionally in the sense and/or both antisense and sense strands, are resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi. Non-limiting examples of nucleotides having a northern configuration include locked nucleic acid (LNA) nucleotides (e.g., 2'-O, 4'-C-methylene-(D-ribofuranosyl) nucleotides); 2'-methoxyethoxy (MOE) nucleotides; 2'-methyl-thio-ethyl, 2'-deoxy-2'-fluoro nucleotides. 2'-deoxy-2'-chloro nucleotides, 2'-azido nucleotides, and 2'-O-methyl nucleotides.

The sense strand of a double stranded siNA molecule may have a terminal cap moiety such as an inverted deoxybasic moiety, at the 3'-end, 5'-end, or both 3' and 5'-ends of the sense strand.

Non-limiting examples of conjugates include conjugates and ligands described in Vargeese, et al., U.S. Application Serial No. 10/427,160, filed April 30, 2003, incorporated by reference herein in its entirety, including the drawings. In another embodiment, the conjugate is covalently attached to the chemically-modified siNA molecule via a biodegradable linker. In one embodiment, the conjugate molecule is attached at the 3'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In another embodiment, the conjugate molecule is attached at the 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In yet another embodiment, the conjugate molecule is attached both the 3'-end and 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule, or any combination thereof. In one embodiment, a conjugate molecule of the invention comprises a molecule that facilitates delivery of a chemically-modified siNA molecule into a biological

system, such as a cell. In another embodiment, the conjugate molecule attached to the chemically-modified siNA molecule is a poly ethylene glycol, human serum albumin, or a ligand for a cellular receptor that can mediate cellular uptake. Examples of specific conjugate molecules contemplated by the instant invention that can be attached to chemically-modified siNA molecules are described in Vargeese et al., U.S. Patent Application Publication No. 20030130186, published July 10, 2003, and U.S. Patent Application Publication No. 20040110296, published June 10, 2004. The type of conjugates used and the extent of conjugation of siNA molecules of the invention can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of siNA constructs while at the same time maintaining the ability of the siNA to mediate RNAi activity. As such, one skilled in the art can screen siNA constructs that are modified with various conjugates to determine whether the siNA conjugate complex possesses improved properties while maintaining the ability to mediate RNAi, for example in animal models as are generally known in the art.

A siNA further may be further comprised of a nucleotide, non-nucleotide, or mixed nucleotide/non-nucleotide linker that joins the sense region of the siNA to the antisense region of the siNA. In one embodiment, a nucleotide linker can be a linker of >2 nucleotides in length, for example about 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. In another embodiment, the nucleotide linker can be a nucleic acid aptamer. By "aptamer" or "nucleic acid aptamer" as used herein is meant a nucleic acid molecule that binds specifically to a target molecule wherein the nucleic acid molecule has sequence that comprises a sequence recognized by the target molecule in its natural setting. Alternately, an aptamer can be a nucleic acid molecule that binds to a target molecule where the target molecule does not naturally bind to a nucleic acid. The target molecule can be any molecule of interest. For example, the aptamer can be used to bind to a ligand-binding domain of a protein, thereby preventing interaction of the naturally occurring ligand with the protein. This is a non-limiting example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art. See, for example, Gold, et al., *Annu. Rev. Biochem. 64*:763, 1995; Brody and Gold, *J. Biotechnol. 74*:5, 2000; Sun, *Curr. Opin. Mol. Ther. 2*:100, 2000; Kusser, *J. Biotechnol. 74*:27, 2000; Hermann and Patel, *Science 287*:820, 2000; and Jayasena, *Clinical Chemistry 45*:1628, 1999.

A non-nucleotide linker may be comprised of an abasic nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, or other polymeric compounds (e.g., polyethylene glycols such as those having between 2 and 100 ethylene glycol units). Specific examples include those described by Seela and Kaiser, *Nucleic Acids Res. 18*:6353, 1990, and *Nucleic Acids Res. 15*:3113, 1987; Cload and Schepartz, *J. Am. Chem. Soc. 113*:6324, 1991;

Richardson and Schepartz, *J. Am. Chem. Soc. 113*:5109, 1991; Ma, et al., *Nucleic Acids Res. 21*:2585, 1993, and *Biochemistry 32*:1751, 1993; Durand, et al., *Nucleic Acids Res. 18*:6353, 1990; McCurdy, et al., *Nucleosides & Nucleotides 10*:287, 1991; Jschke, et al., *Tetrahedron Lett. 34*:301, 1993; Ono, et al., *Biochemistry 30*:9914, 1991; Arnold, et al., International Publication No. WO 89/02439; Usman, et al., International Publication No. WO 95/06731; Dudycz, et al., International Publication No. WO 95/11910 and Ferentz and Verdine, *J. Am. Chem. Soc. 113*:4000, 1991. A "non-nucleotide" further means any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound can be abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymidine, for example at the C1 position of the sugar.

The synthesis of a siNA molecule of the invention, which can be chemically-modified, comprises: (a) synthesis of two complementary strands of the siNA molecule; (b) annealing the two complementary strands together under conditions suitable to obtain a double-stranded siNA molecule. In another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase oligonucleotide synthesis. In yet another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase tandem oligonucleotide synthesis.

Oligonucleotides (e.g., certain modified oligonucleotides or portions of oligonucleotides lacking ribonucleotides) are synthesized using protocols known in the art, for example as described in Caruthers, et al., *Methods in Enzymology 211*:3-19, 1992; Thompson, et al., International PCT Publication No. WO 99/54459; Wincott, et al., *Nucleic Acids Res. 23*:2677-2684, 1995; Wincott, et al., *Methods Mol. Bio. 74*:59, 1997; Brennan, et al., *Biotechnol Bioeng. 61*:33-45, 1998; and Brennan, U.S. Patent No. 6,001,311. Synthesis of RNA, including certain siNA molecules of the invention, follows general procedures as described, for example, in Usman, et al., *J. Am. Chem. Soc. 109*:7845, 1987; Scaringe, et al., *Nucleic Acids Res. 18*:5433, 1990; and Wincott, et al., *Nucleic Acids Res. 23*:2677-2684, 1995; Wincott, et al., *Methods Mol. Bio. 74*:59, 1997.

Supplemental or complementary methods for delivery of nucleic acid molecules for use within then invention are described, for example, in Akhtar, et al., *Trends Cell Bio. 2*:139, 1992; *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, ed. Akhtar, 1995; Maurer, et al., *Mol. Membr. Biol. 16*:129-140, 1999; Hofland and Huang, *Handb. Exp. Pharmacol. 137*:165-192, 1999; and Lee, et al., *ACS Symp. Ser. 752*:184-192, 2000. Sullivan, et al.,

International PCT Publication No WO 94/02595, further describes general methods for delivery of enzymatic nucleic acid molecules. These protocols can be utilized to supplement or complement delivery of virtually any nucleic acid molecule contemplated within the invention.

Nucleic acid molecules and polynucleotide delivery-enhancing polypeptides can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, administration within formulations that comprise the siNA and polynucleotide delivery-enhancing polypeptide alone, or that further comprise one or more additional components, such as a pharmaceutically acceptable carrier, diluent, excipient, adjuvant, emulsifier, buffer, stabilizer, preservative, and the like. In certain embodiments, the siNA and/or the polynucleotide delivery-enhancing polypeptide can be encapsulated in liposomes, administered by iontophoresis, or incorporated into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, bioadhesive microspheres, or proteinaceous vectors (see e.g., O'Hare and Normand, International PCT Publication No. WO 00/53722). Alternatively, a nucleic acid/peptide/vehicle combination can be locally delivered by direct injection or by use of an infusion pump. Direct injection of the nucleic acid molecules of the invention, whether subcutaneous, intramuscular, or intradermal, can take place using standard needle and syringe methodologies, or by needle-free technologies such as those described in Conry, et al., *Clin. Cancer Res.* 5:2330-2337, 1999, and Barry, et al., International PCT Publication No. WO 99/31262.

The compositions of the instant invention can be effectively employed as pharmaceutical agents. Pharmaceutical agents prevent, modulate the occurrence or severity of, or treat (alleviate one or more symptom(s) to a detectable or measurable extent) of a disease state or other adverse condition in a patient.

Thus within additional embodiments the invention provides pharmaceutical compositions and methods featuring the presence or administration of one or more polynucleic acid(s), typically one or more siNAs, combined, complexed, or conjugated with a polynucleotide delivery-enhancing polypeptide, optionally formulated with a pharmaceutically-acceptable carrier, such as a diluent, stabilizer, buffer, and the like.

The present invention satisfies additional objects and advantages by providing short interfering nucleic acid (siNA) molecules that modulate expression of genes associated with a particular disease state or other adverse condition in a subject. Typically, the siNA will target a gene that is expressed at an elevated level as a causal or contributing factor associated with the subject disease state or adverse condition. In this context, the siNA will effectively downregulate expression of the gene to levels that prevent, alleviate, or reduce the severity or

recurrence of one or more associated disease symptoms. Alternatively, for various distinct disease models where expression of the target gene is not necessarily elevated as a consequence or sequel of disease or other adverse condition, down regulation of the target gene will nonetheless result in a therapeutic result by lowering gene expression (i.e., to reduce levels of a selected mRNA and/or protein product of the target gene). Alternatively, siNAs of the invention may be targeted to lower expression of one gene, which can result in upregulation of a "downstream" gene whose expression is negatively regulated by a product or activity of the target gene.

Within exemplary embodiments, the compositions and methods of the invention are useful as therapeutic tools to regulate expression of tumor necrosis factor–α (TNF-α) to treat or prevent symptoms of rheumatoid arthritis (RA). In this context the invention further provides compounds, compositions, and methods useful for modulating expression and activity of TNF- α by RNA interference (RNAi) using small nucleic acid molecules. In more detailed embodiments, the invention provides small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (mRNA), and short hairpin RNA (shRNA) molecules, and related methods, that are effective for modulating expression of TNF-α and/or TNF-α genes to prevent or alleviate symptoms of RA in mammalian subjects. Within these and related therapeutic compositions and methods, the use of chemically-modified siNAs will often improve properties of the modified siNAs in comparison to properties of native siNA molecules, for example by providing increased resistance to nuclease degradation in vivo, and/or through improved cellular uptake. As can be readily determined according to the disclosure herein, useful siNAs having multiple chemical modifications will retain their RNAi activity. The siNA molecules of the instant invention thus provide useful reagents and methods for a variety of therapeutic, diagnostic, target validation, genomic discovery, genetic engineering, and pharmacogenomic applications.

This siNAs of the present invention may be administered in any form, for example transdermally or by local injection (e.g., local injection at sites of psoriatic plaques to treat psoriasis, or into the joints of patients afflicted with psoriatic arthritis or RA). In more detailed embodiments, the invention provides formulations and methods to administer therapeutically effective amounts of siNAs directed against of a mRNA of TNF-α, which effectively down-regulate the TNF- α RNA and thereby reduce or prevent one or more TNF-α-associated inflammatory condition(s). Comparable methods and compositions are provided that target expression of one or more different genes associated with a selected disease condition in animal

subjects, including any of a large number of genes whose expression is known to be aberrantly increased as a causal or contributing factor associated with the selected disease condition.

The siNA/polynucleotide delivery-enhancing polypeptide mixtures of the invention can be administered in conjunction with other standard treatments for a targeted disease condition, for example in conjunction with therapeutic agents effective against inflammatory diseases, such as RA or psoriasis. Examples of combinatorially useful and effective agents in this context include non-steroidal antiinflammatory drugs (NSAIDs), methotrexate, gold compounds, D-penicillamine, the antimalarials, sulfasalazine, glucocorticoids, and other TNF-α neutralizing agents such as infliximab and entracept.

Negatively charged polynucleotides of the invention (e.g., RNA or DNA) can be administered to a patient by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The compositions of the present invention may also be formulated and used as tablets, capsules or elixirs for oral administration, suppositories for rectal administration, sterile solutions, suspensions for injectable administration, and the other compositions known in the art.

The present invention also includes pharmaceutically acceptable formulations of the compositions described herein. These formulations include salts of the above compounds, e.g., acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, e.g., systemic administration, into a cell or patient, including for example a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation from reaching a target cell (i.e., a cell to which the negatively charged nucleic acid is desirable for delivery). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity.

By "systemic administration" is meant in vivo systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include, without limitation: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes exposes the desired negatively charged polymers, e.g., nucleic acids, to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function

of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation that can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach may provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells, such as cancer cells.

By "pharmaceutically acceptable formulation" is meant, a composition or formulation that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity. Non-limiting examples of agents suitable for formulation with the nucleic acid molecules of the instant invention include: P-glycoprotein inhibitors (such as Pluronic P85), which can enhance entry of drugs into the CNS (Jolliet-Riant and Tillement, *Fundam. Clin. Pharmacol. 13*:16-26, 1999); biodegradable polymers, such as poly (DL-lactide-coglycolide) microspheres for sustained release delivery after intracerebral implantation (Emerich, D.F., et al., *Cell Transplant 8*:47-58, 1999) (Alkermes, Inc. Cambridge, Mass.); and loaded nanoparticles, such as those made of polybutylcyanoacrylate, which can deliver drugs across the blood brain barrier and can alter neuronal uptake mechanisms (*Prog Neuropsychopharmacol Biol Psychiatry 23*:941-949, 1999). Other non-limiting examples of delivery strategies for the nucleic acid molecules of the instant invention include material described in Boado, et al., *J. Pharm. Sci. 87*:1308-1315, 1998; Tyler, et al., *FEBS Lett. 421*:280-284, 1999; Pardridge, et al., *PNAS USA. 92*:5592-5596, 1995; Boado, *Adv. Drug Delivery Rev. 15*:73-107, 1995; Aldrian-Herrada, et al., *Nucleic Acids Res. 26*:4910-4916, 1998; and Tyler, et al., *PNAS USA. 96*:7053-7058, 1999.

The present invention also includes compositions prepared for storage or administration, which include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., A.R. Gennaro ed., 1985. For example, preservatives, stabilizers, dyes and flavoring agents may be provided. These include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. In addition, antioxidants and suspending agents may be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence of, or treat (alleviate a symptom to some extent, preferably all of the symptoms) a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the

route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors that those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer.

Aqueous suspensions contain the active materials in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropyl-methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents can be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions can also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.

Oily suspensions can be formulated by suspending the active ingredients in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions can contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents can be added to provide palatable oral preparations. These compositions can be preserved by the addition of an anti-oxidant such as ascorbic acid.

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents or suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, can also be present.

Pharmaceutical compositions of the invention can also be in the form of oil-in-water emulsions. The oily phase can be a vegetable oil or a mineral oil or mixtures of these. Suitable emulsifying agents can be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, for example sorbitan monooleate, and

condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions can also contain sweetening and flavoring agents.

The pharmaceutical compositions can be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents that have been mentioned above. The sterile injectable preparation can also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be employed including synthetic mono-or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The siNAs can also be administered in the form of suppositories, e.g., for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable non-irritating excipient that is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials include cocoa butter and polyethylene glycols.

The siNAs can be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-H. For a review see Usman and Cedergren, *TIBS 17*:34, 1992; Usman, et al., *Nucleic Acids Symp. Ser. 31*:163, 1994. SiNA constructs can be purified by gel electrophoresis using general methods or can be purified by high pressure liquid chromatography and re-suspended in water.

Chemically synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) can prevent their degradation by serum ribonucleases, which can increase their potency. See e.g.; Eckstein, et al., International Publication No. WO 92/07065; Perrault, et al., *Nature 344*:565, 1990; Pieken, et al., *Science 253*:314, 1991; Usman and Cedergren, *Trends in Biochem. Sci. 17*:334, 1992; Usman, et al., International Publication No. WO 93/15187; and Rossi, et al., International Publication No. WO 91/03162; Sproat, U.S. Patent No. 5,334,711; Gold, et al., U.S. Patent No. 6,300,074. All of the above references describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of the nucleic acid molecules described herein.

There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease

stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-fluoro, 2'-O-methyl, 2'-O-allyl, 2'-H, nucleotide base modifications. For a review see Usman and Cedergren, *TIBS 17*:34, 1992; Usman, et al., *Nucleic Acids Symp. Ser. 31*:163, 1994; Burgin, et al., *Biochemistry 35*:14090, 1996. Sugar modification of nucleic acid molecules have been extensively described in the art. See Eckstein, et al., International Publication PCT No. WO 92/07065; Perrault, et al. *Nature 344*:565-568, 1990; Pieken, et al., *Science 253*:314-317, 1991; Usman and Cedergren, *Trends in Biochem. Sci. 17*:334-339, 1992; Usman, et al. International Publication PCT No. WO 93/15187; Sproat, U.S. Patent No. 5,334,711 and Beigelman, et al., *J. Biol. Chem. 270*:25702, 1995; Beigelman, et al., International PCT Publication No. WO 97/26270; Beigelman, et al., U.S. Patent No. 5,716,824; Usman, et al., U.S. Patent No. 5,627,053; Woolf, et al., International PCT Publication No. WO 98/13526; Thompson, et al., Karpeisky, et al., *Tetrahedron Lett. 39*:1131, 1998; Earnshaw and Gait, *Biopolymers (Nucleic Acid Sciences) 48*:39-55, 1998; Verma and Eckstein, *Annu. Rev. Biochem. 67*:99-134, 1998; and Burlina, et al., *Bioorg. Med. Chem. 5*:1999-2010, 1997. Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into nucleic acid molecules without modulating catalysis. In view of such teachings, similar modifications can be used as described herein to modify the siNA nucleic acid molecules of the instant invention so long as the ability of siNA to promote RNAi in cells is not significantly inhibited.

While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorodithioate, and/or 5'-methylphosphonate linkages improves stability, excessive modifications can cause some toxicity or decreased activity. Therefore, when designing nucleic acid molecules, the amount of these internucleotide linkages should be minimized. The reduction in the concentration of these linkages should lower toxicity, resulting in increased efficacy and higher specificity of these molecules.

In one embodiment, the invention features modified siNA molecules, with phosphate backbone modifications comprising one or more phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and/or alkylsilyl, substitutions. For a review of oligonucleotide backbone modifications, see Hunziker and Leumann, "Nucleic Acid Analogues: Synthesis and Properties, in Modern Synthetic Methods," *VCH*, 1995, pp. 331-417, and Mesmaeker, et al., "Novel Backbone Replacements for Oligonucleotides, in Carbohydrate Modifications in Antisense Research," *ACS*, 1994, pp. 24-39.

Methods for the delivery of nucleic acid molecules are described in Akhtar, et al., *Trends Cell Bio. 2*:139, 1992; *Delivery Strategies for Antisense Oligonucleotide Therapeutics,* ed. Akhtar, 1995; Maurer, et al., *Mol. Membr. Biol. 16*:129-140, 1999; Hofland and Huang, *Handb. Exp. Pharmacol. 137*:165-192, 1999; and Lee, et al., *ACS Symp. Ser. 752*:184-192, 2000. Beigelman, et al., U.S. Patent No. 6,395,713 and Sullivan, et al., PCT WO 94/02595 further describe the general methods for delivery of nucleic acid molecules. These protocols can be utilized for the delivery of virtually any nucleic acid molecule. Nucleic acid molecules can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as biodegradable polymers, hydrogels, cyclodextrins (see for example, Gonzalez, et al., *Bioconjugate Chem. 10*:1068-1074, 1999; Wang, et al., International PCT Publication Nos. WO 03/47518 and WO 03/46185), poly(lactic-co-glycolic)ac- id (PLGA) and PLCA microspheres (see for example, U.S. Patent No. 6,447,796 and U.S. Patent Application Publication No. US 2002130430), biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors (O'Hare and Normand, International PCT Publication No. WO 00/53722). Alternatively, the nucleic acid/vehicle combination is locally delivered by direct injection or by use of an infusion pump. Direct injection of the nucleic acid molecules of the invention, whether subcutaneous, intramuscular, or intradermal, can take place using standard needle and syringe methodologies, or by needle-free technologies such as those described in Conry, et al., *Clin. Cancer Res. 5*:2330-2337, 1999, and Barry, et al., International PCT Publication No. WO 99/31262. The molecules of the instant invention can be used as pharmaceutical agents. Pharmaceutical agents prevent, modulate the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state in a subject.

The term "ligand" refers to any compound or molecule, such as a drug, peptide, hormone, or neurotransmitter, that is capable of interacting with another compound, such as a receptor, either directly or indirectly. The receptor that interacts with a ligand can be present on the surface of a cell or can alternately be an intracellular receptor. Interaction of the ligand with the receptor can result in a biochemical reaction, or can simply be a physical interaction or association.

By "asymmetric hairpin" as used herein is meant a linear siNA molecule comprising an antisense region, a loop portion that can comprise nucleotides or non-nucleotides, and a sense region that comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complementary nucleotides to base pair with the antisense region and form a

duplex with loop. For example, an asymmetric hairpin siNA molecule of the invention can comprise an antisense region having length sufficient to mediate RNAi in a T-cell (e.g., about 19 to about 22 (e.g., about 19, 20, 21, or 22) nucleotides and a loop region comprising about 4 to about 8 (e.g., about 4, 5, 6, 7, or 8) nucleotides, and a sense region having about 3 to about 18 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18) nucleotides that are complementary to the antisense region. The asymmetric hairpin siNA molecule can also comprise a 5'-terminal phosphate group that can be chemically modified. The loop portion of the asymmetric hairpin siNA molecule can comprise nucleotides, non-nucleotides, linker molecules, or conjugate molecules as described herein.

By "asymmetric duplex" as used herein is meant a siNA molecule having two separate strands comprising a sense region and an antisense region, wherein the sense region comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complementary nucleotides to base pair with the antisense region and form a duplex. For example, an asymmetric duplex siNA molecule of the invention can comprise an antisense region having length sufficient to mediate RNAi in a T-cell (e.g., about 19 to about 22 (e.g., about 19, 20, 21, or 22) nucleotides and a sense region having about 3 to about 18 (e.g., about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18) nucleotides that are complementary to the antisense region.

By "modulate gene expression" is meant that the expression of a target gene is upregulated or downregulated, which can include upregulation or downregulation of mRNA levels present in a cell, or of mRNA translation, or of synthesis of protein or protein subunits, encoded by the target gene. Modulation of gene expression can be determined also be the presence, quantity, or activity of one or more proteins or protein subunits encoded by the target gene that is up regulated or down regulated, such that expression, level, or activity of the subject protein or subunit is greater than or less than that which is observed in the absence of the modulator (e.g., a siRNA). For example, the term "modulate" can mean "inhibit," but the use of the word "modulate" is not limited to this definition.

By "inhibit", "down-regulate", or "reduce" expression, it is meant that the expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more proteins or protein subunits, or level or activity of one or more proteins or protein subunits encoded by a target gene, is reduced below that observed in the absence of the nucleic acid molecules (e.g., siNA) of the invention. In one embodiment, inhibition, down-regulation or reduction with an siNA molecule is below that level observed in the presence of an inactive or attenuated molecule. In another embodiment, inhibition, down-regulation, or reduction with

siNA molecules is below that level observed in the presence of, for example, an siNA molecule with scrambled sequence or with mismatches. In another embodiment, inhibition, down-regulation, or reduction of gene expression with a nucleic acid molecule of the instant invention is greater in the presence of the nucleic acid molecule than in its absence.

Gene "silencing" refers to partial or complete loss-of-function through targeted inhibition of gene expression in a cell and may also be referred to as "knock down." Depending on the circumstances and the biological problem to be addressed, it may be preferable to partially reduce gene expression. Alternatively, it might be desirable to reduce gene expression as much as possible. The extent of silencing may be determined by methods known in the art, some of which are summarized in International Publication No. WO 99/32619. Depending on the assay, quantification of gene expression permits detection of various amounts of inhibition that may be desired in certain embodiments of the invention, including prophylactic and therapeutic methods, which will be capable of knocking down target gene expression, in terms of mRNA levels or protein levels or activity, for example, by equal to or greater than 10%, 30%, 50%, 75% 90%, 95% or 99% of baseline (i.e., normal) or other control levels, including elevated expression levels as may be associated with particular disease states or other conditions targeted for therapy.

The phrase "inhibiting expression of a target gene" refers to the ability of a siNA of the invention to initiate gene silencing of the target gene. To examine the extent of gene silencing, samples or assays of the organism of interest or cells in culture expressing a particular construct are compared to control samples lacking expression of the construct. Control samples (lacking construct expression) are assigned a relative value of 100%. Inhibition of expression of a target gene is achieved when the test value relative to the control is about 90%, often 50%, and in certain embodiments 25-0%. Suitable assays include, e.g., examination of protein or mRNA levels using techniques known to those of skill in the art such as dot blots, northern blots, *in situ* hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

By "subject" is meant an organism, tissue, or cell, which may include an organism as the subject or as a donor or recipient of explanted cells or the cells that are themselves subjects for siNA delivery. "Subject" therefore may refers to an organism, organ, tissue, or cell, including in vitro or ex vivo organ, tissue or cellular subjects, to which the nucleic acid molecules of the invention can be administered and enhanced by polynucleotide delivery-enhancing polypeptides described herein. Exemplary subjects include mammalian individuals or cells, for example human patients or cells.

As used herein "cell" is used in its usual biological sense, and does not refer to an entire multicellular organism, e.g., specifically does not refer to a human. The cell can be present in an organism, e.g., birds, plants and mammals such as humans, cows, sheep, apes, monkeys, swine, dogs, and cats. The cell can be prokaryotic (e.g., bacterial cell) or eukaryotic (e.g., mammalian or plant cell). The cell can be of somatic or germ line origin, totipotent or pluripotent, dividing or non-dividing. The cell can also be derived from or can comprise a gamete or embryo, a stem cell, or a fully differentiated cell.

By "vectors" is meant any nucleic acid- and/or viral-based technique used to deliver a desired nucleic acid.

By "comprising" is meant including, but not limited to, whatever follows the word "comprising." Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of." Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

By "RNA" is meant a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" is meant a nucleotide with a hydroxyl group at the 2' position of a .beta.-D-ribo-furanose moiety. The terms include double-stranded RNA, single-stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in the RNA molecules of the instant invention can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

By "highly conserved sequence region" is meant, a nucleotide sequence of one or more regions in a target gene does not vary significantly from one generation to the other or from one biological system to the other.

By "sense region" is meant a nucleotide sequence of a siNA molecule having complementarity to an antisense region of the siNA molecule. In addition, the sense region of a siNA molecule can comprise a nucleic acid sequence having homology with a target nucleic acid sequence.

By "antisense region" is meant a nucleotide sequence of a siNA molecule having complementarity to a target nucleic acid sequence. In addition, the antisense region of a siNA molecule can optionally comprise a nucleic acid sequence having complementarity to a sense region of the siNA molecule.

By "target nucleic acid" is meant any nucleic acid sequence whose expression or activity is to be modulated. The target nucleic acid can be DNA or RNA.

By "complementarity" is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types. In reference to the nucleic molecules of the present invention, the binding free energy for a nucleic acid molecule with its complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., RNAi activity. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner, et al., *CSH Symp. Quant. Biol.*, LII, 1987, pp. 123-133; Frier, et al., *Proc. Nat. Acad. Sci. USA 83*:9373-9377, 1986; Turner, et al., *J. Am. Chem. Soc. 109*:3783-3785, 1987. A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, or 10 nucleotides out of a total of 10 nucleotides in the first oligonucleotide being based paired to a second nucleic acid sequence having 10 nucleotides represents 50%, 60%, 70%, 80%, 90%, and 100% complementary respectively). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence.

The term "universal base" as used herein refers to nucleotide base analogs that form base pairs with each of the natural DNA/RNA bases with little discrimination between them. Non-limiting examples of universal bases include C-phenyl, C-naphthyl and other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole as known in the art (see for example, Loakes, *Nucleic Acids Research 29*:2437-2447, 2001).

The term "acyclic nucleotide" as used herein refers to any nucleotide having an acyclic ribose sugar, for example where any of the ribose carbons (C1, C2, C3, C4, or C5), are independently or in combination absent from the nucleotide.

The term "biodegradable" as used herein, refers to degradation in a biological system, for example enzymatic degradation or chemical degradation.

The term "biologically active molecule" as used herein, refers to compounds or molecules that are capable of eliciting or modifying a biological response in a system. Non-limiting examples of biologically active siNA molecules either alone or in combination with other molecules contemplated by the instant invention include therapeutically active molecules such as antibodies, cholesterol, hormones, antivirals, peptides, proteins, chemotherapeutics, small molecules, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, 2,5-A chimeras, siNA, dsRNA, allozymes, aptamers, decoys and analogs thereof. Biologically active molecules of the invention also include molecules capable of modulating the pharmacokinetics and/or pharmacodynamics of other biologically active molecules, for example, lipids and polymers such as polyamines, polyamides, polyethylene glycol and other polyethers.

The term "phospholipid" as used herein, refers to a hydrophobic molecule comprising at least one phosphorus group. For example, a phospholipid can comprise a phosphorus-containing group and saturated or unsaturated alkyl group, optionally substituted with OH, COOH, oxo, amine, or substituted or unsubstituted aryl groups.

By "cap structure" is meant chemical modifications, which have been incorporated at either terminus of the oligonucleotide (see, for example, Adamic, et al., U.S. Patent No. 5,998,203, incorporated by reference herein). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and may help in delivery and/or localization within a cell. The cap may be present at the 5'-terminus (5'-cap) or at the 3'-terminal (3'-cap) or may be present on both termini. In non-limiting examples, the 5'-cap includes, but is not limited to, glyceryl, inverted deoxy abasic residue (moiety); 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide, 4'-thio nucleotide; carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; threo-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety.

Non-limiting examples of the 3'-cap include, but are not limited to, glyceryl, inverted deoxy abasic residue (moiety), 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl)

nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate; 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; threo-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Lyer, *Tetrahedron 49*:1925, 1993; incorporated by reference herein).

By the term "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine and therefore lacks a base at the 1'-position.

By "nucleotide" as used herein is as recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see, for example, Usman and McSwiggen, *supra*; Eckstein, et al., International PCT Publication No. WO 92/07065; Usman, et al., International PCT Publication No. WO 93/15187; Uhlman & Peyman, *supra*, all are hereby incorporated by reference herein. There are several examples of modified nucleic acid bases known in the art as summarized by Limbach, et al., *Nucleic Acids Res. 22*:2183, 1994. Some of the non-limiting examples of base modifications that can be introduced into nucleic acid molecules include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (e.g., 5-methylcytidine), 5-alkyluridines (e.g., ribothymidine), 5-halouridine (e.g., 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (e.g. 6-methyluridine), propyne, and others (Burgin, et al., *Biochemistry 35*:14090, 1996; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents.

By "target site" is meant a sequence within a target RNA that is "targeted" for cleavage mediated by a siNA construct which contains sequences within its antisense region that are complementary to the target sequence.

By "detectable level of cleavage" is meant cleavage of target RNA (and formation of cleaved product RNAs) to an extent sufficient to discern cleavage products above the background of RNAs produced by random degradation of the target RNA. Production of cleavage products from 1-5% of the target RNA is sufficient to detect above the background for most methods of detection.

By "biological system" is meant, material, in a purified or unpurified form, from biological sources, including but not limited to human, animal, plant, insect, bacterial, viral or other sources, wherein the system comprises the components required for RNAi activity. The term "biological system" includes, for example, a cell, tissue, or organism, or extract thereof. The term biological system also includes reconstituted RNAi systems that can be used in an in vitro setting.

The term "biodegradable linker" as used herein, refers to a nucleic acid or non-nucleic acid linker molecule that is designed as a biodegradable linker to connect one molecule to another molecule, for example, a biologically active molecule to a siNA molecule of the invention or the sense and antisense strands of a siNA molecule of the invention. The biodegradable linker is designed such that its stability can be modulated for a particular purpose, such as delivery to a particular tissue or cell type. The stability of a nucleic acid-based biodegradable linker molecule can be modulated by using various chemistries, for example combinations of ribonucleotides, deoxyribonucleotides, and chemically-modified nucleotides, such as 2'-O-methyl, 2'-fluoro, 2'-amino, 2'-O-amino, 2'-C-allyl, 2'-O-allyl, and other 2'-modified or base modified nucleotides. The biodegradable nucleic acid linker molecule can be a dimer, trimer, tetramer or longer nucleic acid molecule, for example, an oligonucleotide of about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length, or can comprise a single nucleotide with a phosphorus-based linkage, for example, a phosphoramidate or phosphodiester linkage. The biodegradable nucleic acid linker molecule can also comprise nucleic acid backbone, nucleic acid sugar, or nucleic acid base modifications.

By "abasic" is meant sugar moieties lacking a base or having other chemical groups in place of a base at the 1' position, see for example Adamic, et al., U.S. Patent No. 5,998,203.

By "unmodified nucleoside" is meant one of the bases adenine, cytosine, guanine, thymine, or uracil joined to the 1' carbon of beta.-D-ribo-furanose.

By "modified nucleoside" is meant any nucleotide base which contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate. Non-limiting examples of modified nucleotides are shown by Formulae I-VII and/or other modifications described herein.

In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant 2'-NH_2 or 2'-O--NH_2, which can be modified or unmodified. Such modified groups are described, for example, in Eckstein et al., U.S. Patent No. 5,672,695 and Matulic-Adamic, et al., U.S. Patent. No. 6,248,878.

The siNA molecules can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. The nucleic acid or nucleic acid complexes can be locally administered to through injection, infusion pump or stent, with or without their incorporation in biopolymers. In another embodiment, polyethylene glycol (PEG) can be covalently attached to siNA compounds of the present invention, to the polynucleotide delivery-enhancing polypeptide, or both. The attached PEG can be any molecular weight, preferably from about 2,000 to about 50,000 daltons (Da).

The sense region can be connected to the antisense region via a linker molecule, such as a polynucleotide linker or a non-nucleotide linker.

"Inverted repeat" refers to a nucleic acid sequence comprising a sense and an antisense element positioned so that they are able to form a double stranded siRNA when the repeat is transcribed. The inverted repeat may optionally include a linker or a heterologous sequence such as a self-cleaving ribozyme between the two elements of the repeat. The elements of the inverted repeat have a length sufficient to form a double stranded RNA. Typically, each element of the inverted repeat is about 15 to about 100 nucleotides in length, preferably about 20-30 base nucleotides, preferably about 20-25 nucleotides in length, e.g., 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

"Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

"Large double-stranded RNA" refers to any double-stranded RNA having a size greater than about 40 base pairs (bp) for example, larger than 100 bp or more particularly larger than 300 bp. The sequence of a large dsRNA may represent a segment of an mRNA or the entire mRNA. The maximum size of the large dsRNA is not limited herein. The double-stranded RNA may include modified bases where the modification may be to the phosphate sugar backbone or to the nucleoside. Such modifications may include a nitrogen or sulfur heteroatom or any other modification known in the art.

The double-stranded structure may be formed by self-complementary RNA strand such as occurs for a hairpin or a micro RNA or by annealing of two distinct complementary RNA strands.

"Overlapping" refers to when two RNA fragments have sequences which overlap by a plurality of nucleotides on one strand, for example, where the plurality of nucleotides (nt) numbers as few as 2-5 nucleotides or by 5-10 nucleotides or more.

"One or more dsRNAs" refers to dsRNAs that differ from each other on the basis of sequence.

"Target gene or mRNA" refers to any gene or mRNA of interest. Indeed any of the genes previously identified by genetics or by sequencing may represent a target. Target genes or mRNA may include developmental genes and regulatory genes as well as metabolic or structural genes or genes encoding enzymes. The target gene may be expressed in those cells in which a phenotype is being investigated or in an organism in a manner that directly or indirectly impacts a phenotypic characteristic. The target gene may be endogenous or exogenous. Such cells include any cell in the body of an adult or embryonic animal or plant including gamete or any isolated cell such as occurs in an immortal cell line or primary cell culture.

In this specification and the appended claims, the singular forms of "a", "an" and "the" include plural reference unless the context clearly dictates otherwise.

EXAMPLES

The above disclosure generally describes the present invention, which is further exemplified by the following examples. These examples are described solely for purposes of illustration, and are not intended to limit the scope of the invention. Although specific terms and values have been employed herein, such terms and values will likewise be understood as exemplary and non-limiting to the scope of the invention.

Example 1

Production and Characterization of Compositions Comprising a siRNA Complexed With a Polynucleotide Delivery-Enhancing Polypeptide

To form complexes between candidate siRNAs and polynucleotide delivery-enhancing polypeptides of the invention, an adequate amount of siRNA is combined with a pre-determined amount of polynucleotide delivery-enhancing polypeptide, for example in Opti-MEM® cell medium (Invitrogen), in defined ratios and incubated at room temperature for about 10-30 min. Subsequently a selected volume, e.g., about 50 µl, of this mixture is brought into contact with target cells and the cells are incubated for a predetermined incubation period, which in the present example was about 2 hr. The siRNA/peptide mixture can optionally include cell culture medium or other additives such as fetal bovine serum. For H3, H4 and H2b, a series of experiments was performed to complex these polynucleotide delivery-enhancing polypeptides with siRNA in different ratios. Generally this was initiated with a 1:0.01 to 1:50 of siRNA/histone ratio. To each well in a 96-well microtiter plate, 40 pm siRNA was added. Each well contained beta-gal cells at 50% confluency. Exemplary optimized ratios for transfection efficiency are shown in Table 2 below.

Transfections were performed with either regular siRNA or siRNA complexed with one of the above-identified histone proteins on 9L/beta-gal cells. The siRNA was designed to specifically knock down beta-galactosidase mRNA, and activities are expressed as percentage of beta-gal activities from control (control cells were transfected using lipofectamine without the polynucleotide delivery-enhancing polypeptide).

Assays for detecting and/or quantifying the efficiency of siRNA delivery are carried out using conventional methods, for example beta-galactosidase assay or flow cytometry methods.

For beta-galactosidase assays, 9L/LacZ cells, a cell line constitutively expressing beta-galactosidase, were used. 9L/LacZ cells are rat gliosarcoma fibroblast cells that constitutively express LacZ and were obtained from ATCC (#CRL-2200). 9L/LacZ cells were grown in Dulbecco's Modified Essential Medium (DMEM) media with a supplement of 1 mM sodium pyruvate, nonessential amino acids, and 20% fetal bovine serum. Cells were cultured at 37°C and 5% CO_2 supplemented with an antibiotic mixture containing 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 mg/ml Fungizone (Invitrogen). The siRNA duplex designed against beta-gal mRNA was chemically synthesized and used with delivery reagents to evaluate knock-down efficiency.

Peptide Synthesis

Peptides were synthesized by solid-phase Fmoc chemistry on CLEAR-amide resin using a Rainin Symphony synthesizer. Coupling steps were performed using 5 equivalents of HCTU and Fmoc amino acid with an excess of N-methylmorpholine for 40 minutes. Fmoc removal was accomplished by treating the peptide resin with 20% piperidine in DMF for two 10 minute cycles. Upon completion of the entire peptide, the Fmoc group was removed with piperidine and washed extensively with DMF. Maleimido modified peptides were prepared by coupling 3.0 equivalents of 3-maleimidopropionic acid and HCTU in the presence of 6 equivalents of N-methylmorpholine to the N-terminus of the peptide resin. The extent of coupling was monitored by the Kaiser test. The peptides were cleaved from the resin by the addition of 10 mL of TFA containing 2.5% water and 2.5 triisopropyl silane followed by gentle agitation at room temperature for 2 hours. The resulting crude peptide was collected by trituration with ether followed by filtration. The crude product was dissolved in Millipore water and lyophilized to dryness. The crude peptide was taken up in 15 mL of water containing 0.05% TFA and 3 mL acetic acid and loaded onto a Zorbax RX-C8 reversed-phase (22 mm ID x 250 mm, 5 μm particle size) through a 5 mL injection loop at a flow rate of 5 mL/min. The purification was accomplished by running a linear AB gradient of 0.1% B/min where solvent A is 0.05% TFA in water and solvent B is 0.05% TFA in acetonitrile. The purified peptides were analyzed by HPLC and ESMS.

siRNA Synthesis and Preparation

Synthesis of oligonucleotides was carried out using the standard 2-cyanoethyl phosphoramidite method on long chain alkylamine controlled pore glass derivatized with 5'-*O*-Dimethyltrityl-2'-*O*-*t*-butyldimethylsilyl-3'-*O*-succinyl ribonucleoside of choice or 5'-*O*-Dimethyltrityl-2'-deoxy-3'-*O*-succinyl thymidine support where applicable. All oligonucleotides were synthesized at either the 0.2 or 1-μmol scale using an ABI 3400 DNA/RNA synthesizer, cleaved from the solid support using concentrated NH_4OH, and deprotected using a 3:1 mixture of NH_4OH : EtOH at 55 °C. The deprotection of 2'-TBDMS protecting groups was achieved by incubating the base-deprotected RNA with a solution (600 μL per μmol) of N-methylpyrrolidone/triethylamine/triethylamine tris(hydrofluoride) (6:3:4 by volume) at 65 °C for 2.5 hours. The corresponding building blocks, 5'-dimethoxytrityl-*N*-(tac)-2'-*O*-(*t*-butyldimethylsilyl)-3'-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidites of A, U, C and G (Proligo, Boulder CO) as well the modified phosphoramidites, 5'DMTr-5-methyl-U-TOM-CE-Phosphoramidite, 5'-DMTr-2'-OMe-Ac-C-CE Phosphoramidite, 5'-DMTr-2'-OMe-G-CE

Phosphoramidite, 5'-DMTr-2'-OMe-U-CE Phosphoramidite, 5'-DMTr-2'-OMe-A-CE Phosphoramidite (Glen Research) were purchased directly from suppliers. Triethylamine-trihydrofluride, *N*-methylpyrrolidinone and concentrated ammonium hydroxide was purchased from Aldrich. All HPLC analysis and purifications were performed on a Waters 2690 with Xterra™ columns. All other reagents were purchased from Glen Research Inc. Oligonucleotides were purified to greater than 97% purity as determined by RP-HPLC. siRNAs for mouse injection were purchased from Qiagen, which were HPLC purified after annealing with acceptable endotoxin level for *in vivo* injection.

Cell Cultures

Primary Human Monocytes:

Fresh human blood samples from healthy donors were purchased from Golden West Biologicals. For isolation of monocytes, blood samples were diluted with PBS at a 1:1 ratio immediately after receiving. Peripheral blood mononuclear cells (PBMC) were first isolated by Ficoll (Amersham) gradient from whole blood. Monocytes were further purified from PBMCs using the Miltenyi CD14 positive selection kit and supplied protocol (MILTENYI BIOTEC). To asses the purity of the monocyte preparation, cells were incubated with an anti-CD14 antibody (BD Biosciences) and then sorted by flow cytometry. The purity of the monocyte preparation was greater than 95%.

Activation of human monocytes was performed by adding 0.1 -1.0 ng /ml of Liposaccharides, LPS (Sigma, St Louis, MO) to the cell culture to stimulate tumor necrosis factor-± (TNF-±) production. Cells were harvested 3 hours after incubation with LPS and mRNA levels were determined by Quantigene assay (Genospectra, Fremont, CA) according to the manufacturer's instructions.

Mouse Tail Fibroblast Cells:

Mouse tail fibroblast (MTF) cells were derived from the tails of C57BL/6J mice. Tails were removed, immersed in 70% ethanol and then cut into small sections with a razor blade. The sections were washed three times with PBS and then incubated in a shaker at 37°C with 0.5 mg/mL collagenase, 100 units/mL penicillin and 100 μg/mL streptomycin to disrupt tissue. Tail sections were then cultured in complete media (Dulbecco's Modified Essential Medium with 20% FBS, 1mM sodium pyruvate, nonessential amino acids and 100 units/mL penicillin and 100 μg/mL streptomycin) until cells were established. Cells were cultured at 37°C, 5% CO_2 in complete media as outlined above.

Transfection Procedure

On the first day of the procedure, saturated 9L/LacZ cultures are taken from T75 flasks, and the cells are detached and diluted into 10 ml of complete medium (DMEM, 1xPS, 1xNa Pyruvate, 1x NEAA). The cells are further diluted to 1:15, and 100 μl of this preparation are aliquoted into wells of 96 well plates, which will generally yield about 50% cell confluence by the next day for the transfection. Edges of the wells are left empty and filled with 250 μl water, and the plates are placed un-stacked in the incubator overnight at 37°C (5% CO_2 incubator).

On the second day, the transfection complex is prepared in Opti-MEM, 50 μl each well. The medium is removed from the plates, and the wells are washed once with 200 μl PBS or Opti-MEM. The plates are blotted and dried completely with tissue by inversion. The transfection mixture is then added (50 μl/well) into each well, and 250 μl water is added to the wells on the edge to prevent them from drying. The cells are then incubated for at least 3 hours at 37°C (5% CO_2 incubator). The transfection mixture is removed and replaced with 100 μl of complete medium (DMEM, 1xPS, 1xNa Pyruvate, 1x NEAA). The cells are cultured for a defined length of time, and then harvested for the enzyme assay.

Cell Viability (MTT Assay)

Cell viability will be assessed using the MTT assay (MTT-100, MatTek kit). This kit measures the uptake and transformation of tetrazolium salt to formazan dye. Thawed and diluted MTT concentrate is prepared 1 hour prior to the end of the dosing period with the lipid by mixing 2 mL of MTT concentrate with 8 mL of MTT diluent. Each cell culture insert is washed twice with PBS containing Ca^{+2} and Mg^{+2} and then transferred to a new 96-well transport plate containing 100 μL of the mixed MTT solution per well. This 96-well transport plate is then incubated for 3 hours at 37°C and 5% CO_2. After the 3 hour incubation, the MTT solution is removed and the cultures are transferred to a second 96-well feeder tray containing 250 μL MTT extractant solution per well. An additional 150 μL of MTT extractan solution was added to the surface of each culture well and the samples sat at room temperature in the dark for a minimum of 2 hours and maximum of 24 hours. The insert membrane was then pierced with a pipet tip and the solutions in the upper and lower wells were allowed to mix. Two hundred microliters of the mixed extracted solution along with extracted blanks (negative control) was transferred to a 96-well plate for measurement with a microplate reader. The optical density (OD) of the samples was measured at 570 nm with the background subtraction at 650 nm on a plate reader. Cell viability was expressed as a percentage and calculated by dividing the OD readings for treated inserts by the OD readings for the PBS treated inserts and multiplying by 100. For the

purposes of this assay, it was assumed that PBS had no effect on cell viability and therefore represented 100% cell viability.

Enzymatic Assay

Reagents for the enzymatic assay were purchased from Invitrogen (β-Gal Assay Kit,), and Fisher (Pierce Micro BCA Protein Assay Reagent Kit, Catalog).

A: Cell Lysis

- Remove the medium, wash once with 200 µl PBS, blot the plate dry with inversion.
- Add 30 µl lysis buffer from β -Gal Kit into each well.
- Freeze-Thaw the cells twice to generate lysate.

B: β -Gal assay

- Prepare assay mix (50 µl 1xbuffer, 17µl ONPG each well)
- Take new plate, add 65 µl assay mix into each well.
- Add 10 µl of cell lysate into each well. There should be blank wells for subtraction of the background activities.
- Incubate at 37°C for about 20 minutes, prevent long incubation which will use up ONPG and bias the high expression.
- Add 100 µl of the Stop solution.
- Measure the OD at 420 nm.

C: BCA assay

- Prepare BSA standard (150 ul per well), points should be duplicated on each plate.
- Put 145 µl of water into each well, add 5 µl of cell lysate into each well.
- Prepare final Assay Reagent according to manufacture's instruction.
- Add 150 µl of Assay Reagent into each well.
- Incubate at 37°C for about 20 minutes.
- Measure the OD at 562 nm.

D: Calculation of specific activity

The specific activity is expressed as nmol of ONPG hydrolyzed/t/mg protein, where t is the time of incubation in minutes at 37°C; mg protein is the protein assayed which is determined by BCA method.

Flow Cytometry Measurement of FITC/FAM Conjugated siRNA

Fluorescence activated cell sorting (FACS) analysis were performed using Beckman Coulter FC500 cell analyzer (Fullerton, CA). The instrument was adjusted according to the

fluorescence probes used (FAM or Cy5 for siRNA and FITC and PE for CD14). Propidium iodide (Fluka, St Lois, MO) and AnnexinV (R&D systems, Minneapolis, MN) were used as indicators for cell viability and cytotoxicity. A brief step-by-step protocol is detailed below.

a) After exposure to the complex of siRNA/peptide, cells were incubated for at least 3 hours.
b) Wash cells with 200 μl PBS.
c) Detach cells with 15 μl TE, incubate at 37°C.
d) Resuspend cells in five wells with 30μl FACS solution (PBS with 0.5% BSA, and 0.1% sodium Azide).
e) Combine all five wells into a tube.
f) Add PI (Propidium iodide) 5 μl into each tube.
g) Analyze the cells with fluorescence activated cell sorting (FCAS) according to manufacturer's instructions.

The siRNA sequence used to silence the beta-galactosidase mRNA was the following:

C.U.A.C.A.C.A.A.A.U.C.A.G.C.G.A.U.U.U.DT.DT (SENSE) (SEQ ID NO: 32)
A.A.A.U.C.G.C.U.G.A.U.U.U.G.U.G.U.A.G.dT.dT (Antisense) (SEQ ID NO: 33)

The data for the present example is shown in Table 2. The transfection efficiency inversely correlates with the amount of beta-galactosidase activity measured from the cell lysate. Upon transfection, a measured decrease in beta-galactosidase activity indicates a successful transfection. Thus, in the absence of transfection, the measured beta-galactosidase activity is 100% and the transfection efficiency is 0%. As beta-galactosidase activity decreases, the transfection efficiency increases. For example, in Table 2, Histone H2B plus siRNA results in a transfection efficiency of 62.03% indicating that the measured beta-galactosidase activity decreased to 37.97%. The same approach for determining transfection efficiency was used for the data presented in Table 3.

Table 2:
Efficiency of siRNA Delivery Mediated by
Polynucleotide Delivery-Enhancing Polypeptides in 9L/LacZ Cells

Transfection Mixture	Transfection Efficiency (% of total cells)	Molar Ratio: (siRNA:Peptide)
siRNA (40 pmol/well) alone	0.09%	
Cationic lipids (Invitrogen)	84.32%	unknown
Histone H2B	62.03%	1:10-15
Histone H3	85.08%	1:10-20
Histone H4	72.07%	1:4-8
GEQIAQLIAGYIDIILKKKKSK (SEQ ID NO: 31)	50.86%	1:5-20
WWETWKPFQCRICMRNFSTRQARRNHRRRHR (SEQ ID NO: 27)	98.29%	1:0.5-4
Poly Lys-Trp, 4:1, MW 20,000-50,000	71.92%	1:2-8
Poly Orn-Trp, 4:1, MW 20,000-50,000	74.16%	1:2-8

siRNA/Peptide/Lipids

To evaluate the effects of adding a cationic lipid to a siRNA/polynucleotide delivery-enhancing polypeptide mixture, complex or conjugate, the above procedures were followed except the lipofectamine (Invitrogen) was added to siRNA/polynucleotide delivery formulation in constant concentrations, following manufacturer's instructions (0.2 μl/ 100 μl Opti-MEM).

To produce the composition comprised of GKINLKALAALAKKIL (SEQ ID NO: 28), siRNA and LIPOFECTIN® (Invitrogen), the siRNA and peptide were mixed together first in Opti-MEM cell culture medium at room temperature, after which LIPOFECTIN® was added at room temperature to the mixture to form the siRNA/peptide/cationic lipid composition.

To produce the composition comprised of RVIRVWFQNKRCKDKK (SEQ ID NO: 29), siRNA and LIPOFECTIN®, the peptide and the LIPOFECTIN® were mixed together first in Opti-MEM cell culture medium, into this mixture was added the siRNA to form the siRNA/peptide/LIPOFECTIN® composition.

To produce the siRNA/peptide/cationic lipid composition using GRKKRRQRRRPPQGRKKRRQRRRPPQGRKKRRQRRRPPQ (SEQ ID NO: 30) or GEQIAQLIAGYIDIILKKKKSK (SEQ ID NO: 31) it does not matter in which order the components are added together to produce the siRNA/peptide/cationic lipid composition.

To produce the siRNA/mellitin/LIPOFECTIN®, the siRNA and mellitin were first mixed together in Opti-MEM cell culture medium and then the LIPOFECTIN® was added to the mixture.

To produce the siRNA/histone H1/LIPOFECTIN® composition, the histone H1 and LIPOFECTIN® were first added together in Opti-MEM cell culture medium thoroughly mixed and then the siRNA was added, thoroughly and mixed with the histone LIPOFECTIN® mixture to form the siRNA/histone H1/ LIPOFECTIN® composition.

Table 3:
Efficiency of siRNA Delivery Mediated by Polynucleotide Delivery-Enhancing Polypeptides With and Without Cationic Lipid in 9L/LacZ Cells

Transfection Mixture	Transfection efficiency with lipids (% of total cells)	Transfection efficiency w/o lipids (% of total cells)	siRNA:Peptide ratio added in transfection mixture
siRNA alone	1.72%	0.11%	
Lipofectamine (no peptide)	83.48%		
GKINLKALAALAKKIL (SEQ ID NO: 28)	89.67%	0.26%	1:5-20
RVIRVWFQNKRCKDKK (SEQ ID NO: 29)	89%	0.59%	1:1-5
GRKKRRQRRRPPQGRKKRRQ RRRPPQGRKKRRQRRRPPQ (SEQ ID NO: 30)	89.99%	54.58%	1:5
GEQIAQLIAGYIDIILKKKKSK (SEQ ID NO: 31)	90.01%	50.86%	1:5-10
Mellitin	93.1%	5.15%	1:20
Histone H1	93.39%	0.14%	1:10-20

Based on the foregoing results, it is apparent that exemplary polynucleotide delivery-enhancing polypeptides of the invention can substantially enhance cellular uptake of siRNAs, while the addition of an optional cationic lipid to certain siRNA/ polynucleotide delivery-enhancing polypeptides mixtures of the invention may substantially improve siRNA delivery efficiency.

EXAMPLE 2

Production and Characterization of Compositions Comprising a siRNA Conjugated With a TAT-HA Polynucleotide Delivery-Enhancing Polypeptide

The present example describes the synthesis and uptake activity of specific peptides covalently conjugated to one strand of a siRNA duplex. These conjugates efficiently deliver siRNA into the cytoplasm.

Peptide Synthesis

Peptides were synthesized by solid-phase Fmoc chemistry on CLEAR-amide resin using a Rainin Symphony synthesizer. Coupling steps were performed using 5 equivalents of HCTU and Fmoc amino acid with an excess of N-methylmorpholine for 40 minutes. Fmoc removal was accomplished by treating the peptide resin with 20% piperidine in DMF for two 10 minutes cycles. Upon completion of the entire peptide, the Fmoc group was removed with piperidine and washed extensively with DMF. Maleimido modified peptides were prepared by coupling 3.0 equivalents of 3-maleimidopropionic acid and HCTU in the presence of 6 equivalents of N-methylmorpholine to the N-terminus of the peptide resin. The extent of coupling was monitored by the Kaiser test. The peptides were cleaved from the resin by the addition of 10 mL of TFA containing 2.5% water and 2.5 triisopropyl silane followed by gentle agitation at room temperature for 2 h. The resulting crude peptide was collected by trituration with ether followed by filtration. The crude product was dissolved in Millipore water and lyophilized to dryness. The crude peptide was taken up in 15 mL of water containing 0.05% TFA and 3 mL acetic acid and loaded onto a Zorbax RX-C8 reversed-phase (22 mm ID x 250 mm, 5 μm particle size) through a 5 mL injection loop at a flow rate of 5 mL/min. The purification was accomplished by running a linear AB gradient of 0.1% B/min where solvent A is 0.05% TFA in water and solvent B is 0.05% TFA in acetonitrile. The purified peptides were analyzed by HPLC and ESMS.

Synthesis of Conjugates

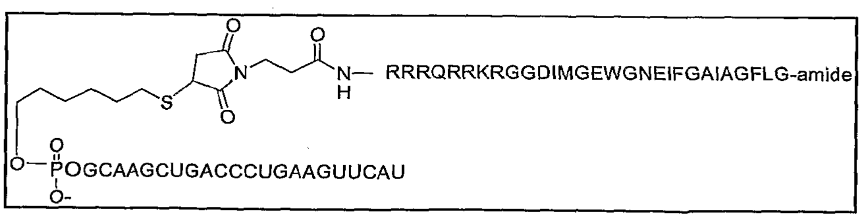
Both peptides and RNAs are prepared using standard solid phase synthesis methods. The peptide and RNA molecules must be functionalized with specific moieties to allow for covalent attachment to each other. For the peptide, the N-terminus is functionalized, for example, with 3-maleimidopropionic acid. However, it is recognized that other functional groups such as bromo or iodoacetyl moieties will work as well. For the RNA molecule the 5' end of the sense strand or 3' end of the antisense strand is functionalized with, for example, a 1-O-dimethoxytrityl-hexyl-disulfide linker according to the following synthetic method.

The 5' modified C6SS-oligonucleotide (GCAAGCUGACCCUGAAGUUCAU (SEQ ID NO: 34); 3.467 mg; 0.4582 μmol) was reduced to the free thiol group with 0.393 mg (3 eq) of tris(2-carboxyethyl)phosphine (TCEP) in 0.3 ml of 0.1 M triethylamine acetate (TEAA) buffer (pH 7.0) at room temperature for 3 h. The reduced oligonucleotide was purified by RP HPLC on XTerra®MS C_{18} 4.6×50mm column using a linear gradient from 0-30% of CH_3CN in 0.1 M TEAA buffer pH 7 within 20 min (t_r=5.931 min).

Purified reduced oligonucleotide (1.361 mg, 0.19085 μmol) was dissolved in 0.2 ml of 0.1 M TEAA buffer pH=7 and then the peptide with the maleimido moiety attached to the peptide N-terminus (0.79 mg, 1.5 eq) was added to the oligonucleotide solution. After addition of peptide a precipitate immediately formed which disappeared upon the addition of 150 μl of 75% CH_3CN/0.1M TEAA. After stirring overnight at room temperature, the resulting conjugate was purified by RP HPLC on XTerra®MS C_{18} 4.6×50mm column using a linear gradient from 0-30% of CH_3CN in 0.1M TEAA buffer pH 7 within 20min and 100% C within next 5 min (t_r=21.007 min). The amount of the conjugate was determined by spectrophotometry based on the calculated molar absorption coefficient at λ=260 nm. MALDI mass spectrometric analysis showed that the peak observed for the conjugate (10 585.3 amu) matches the calculated mass. Yield: 0.509 mg, 0.04815 μmol, 25.2%.

The peptide conjugate sense strand and complimentary antisense strand were annealed in 50 mM potassium acetate, 1 mM magnesium acetate and 15 mM HEPES pH 7.4 by heating at 90°C for 2 min followed by incubation at 37°C for 1 h. The formation of the double stranded RNA conjugate was confirmed by non denaturing (15%) polyacrylamide gel electrophoresis followed by ethidium bromide staining.

Structure of the peptide-siRNA conjugate (SEQ ID NOS 34 and 35)



Uptake Experiments

Cells were plated the day before in 24-well plates so that they were ~50-80% confluent at time of transfection. For complexes, siRNA and peptide were diluted in Opti-MEM® media (Invitrogen), then mixed and allowed to complex 5-10 minutes before adding to cells washed with PBS. Final concentration of siRNA was 500nM at each peptide concentration (2-50 μM). The conjugate, also diluted in Opti-MEM® media, was added to cells at final concentrations ranging from 62.5 nM to 500 nM. At 500nM concentration, we also combined with 20% FBS just before adding to washed cells. Cells were transfected for 3 hours at 37°C, 5%CO_2. Cells were washed with PBS, treated with trypsin and then analyzed by flow cytometry. siRNA

uptake was measured by intensity of Cy5 fluorescence and cellular viability assessed by addition of propidium iodide.

As shown in Figure 1, the peptide/siRNA conjugates achieve a greater percent uptake in mouse tail fibroblast cells than peptide/siRNA complexes. Further, the peptide/siRNA conjugates achieved a higher mean fluorescence intensity (MFI; Figure 2) than the peptide/siRNA compolex. Thus, these data indicate that in certain embodiments it will be desirable to conjugate the polynucleotide delivery-enhancing polypeptide to the siRNA molecule.

EXAMPLE 3

Screening of siRNA/Delivery Peptide Complexes Demonstrates Efficient Induction of siRNA Uptake in 9L/LacZ Cells by a Diverse Assemblage of Rationally-Designed Polynucleotide Delivery-Enhancing Polypeptides

The present example provides additional evidence that a broad and diverse assemblage of rationally-designed polynucleotide delivery-enhancing polypeptides of the invention enhance siRNA uptake when complexed with siRNAs.

Approximately 10,000 9L/lacZ cells were plated per well in flat-bottom 96-well plates so that they would be ~50% confluent the next day at the time of transfection. FAM-labeled siRNA and peptides were diluted in Opti-MEM® media (Invitrogen) at 2-fold the final concentration. Equal volumes of siRNA and peptide were mixed and allowed to complex 5-10 minutes at room temperature and then 50 μL added to cells, previously washed with PBS. Cells were transfected for 3 hours at 37°C, 5%CO_2. Cells were washed with PBS, treated with trypsin and then analyzed by flow cytometry. siRNA uptake was measured by intensity of FAM fluorescence and cellular viability assessed by addition of propidium iodide. Table 4 below summarizes the percent cell uptake data in 9L/LacZ cells for the various rationally-designed polynucleotide delivery-enhancing polypeptides. Included in Table 4, is the concentration of the peptide and siRNA used.

Table 4:

Efficiency of siRNA Uptake Mediated by Rationally-Designed Polynucleotide Delivery-Enhancing Polypeptides 9L/LacZ Cells.

Peptide ID#	Amino Acid Sequence	Peptide Conc.	siRNA Conc.	% Uptake (%PI-/FAM+)
PN173	GRKKRRQRRRPPQC (SEQ ID NO: 36)	10 µM	400 nM	84.8%
PN227	Maleimide-AAVALLPAVLLALLAPRKKRRQRRRPPQ-amide (SEQ ID NO: 37)	1 µM	400 nM	31.0%
PN27	AAVALLPAVLLALLAPRKKRRQRRRPPQC (SEQ ID NO: 38)	1 µM	400 nM	82.6%
PN275	Maleimide- AAVALLPAVLLALLAPRKKRRQRRRPPQ-amide (SEQ ID NO: 37)	4 µM	400 nM	95.3%
PN28	NH2-RKKRRQRRRPPQCAAVALLPAVLLALLAP-amide (SEQ ID NO: 39)	2 µM	400 nM	79.3%
PN69	BrAc-GRKKRRQRRRPQ-amide (SEQ ID NO: 40)	80 µM	400 nM	0.0%
PN81	BrAc-RRRQRRKRGGDIMGEWGNEIFGAIAGFLGamide (SEQ ID NO: 41)	8 µM	800 nM	97.9%
PN250	NH2-RRRQRRKRGGDIMGEWGNEIFGAIAGFLG-amide (SEQ ID NO: 35)	15 µM	800 nM	99.5%
PN204	C(YGRKKRRQRRRG)2 (SEQ ID NO: 42)	1.4 µM	800 nM	82.5%
PN280	Maleimide-GRKKRRQRRRPPQ-amide (SEQ ID NO: 43)	80 µM	400 nM	7.9%
PN350	NH2-KLWKAWPKLWKKLWKP-amide (SEQ ID NO: 44)	10 µM	400 nM	0.0%
PN365	AAVALLPAVLLALLAPRRRRRR-amide (SEQ ID NO: 45)	10 µM	400 nM	81.4%
PN366	RLWRALPRVLRRLLRP-amide (SEQ ID NO: 46)	10 µM	400 nM	0.0%
PN29	NH2-AAVALLPAVLLALLAPSGASGLDKRDYV-amide (SEQ ID NO: 47)	80 µM	400 nM	86.5%
PN235	Maleimide-AAVALLPAVLLALLAPSGASGLDKRDYV-amide (SEQ ID NO: 48)	80 µM	400 nM	0.0%
PN30	NH2-SGASGLDKRDYVAAVAALLPAVLLALLAP-amide (SEQ ID NO: 49)	80 µM	400 nM	0.0%
PN202	NH2-LLETLLKPFQCRICMRNFSTRQARRNHRRRHRR-amide (SEQ ID NO: 50)	2 µM	400 nM	70.8%
PN225	NH2-AAVACRICMRNFSTRQARRNHRRRHRR-amide (SEQ ID NO: 51)	2 µM	400 nM	30.9%
PN236	Maleimide-RQIKIWFQNRRMKWKK-amide (SEQ ID NO: 52)	10 µM	400 nM	37.7%
PN58	RQIKIWFQNRRMKWKK amide (SEQ ID NO: 53)	40 µM	400 nM	75.8%
PN251	NH2-RQIKIWFQNRRMKWKKDIMGEWGNEIFGAIAGFLG-amide (SEQ ID NO: 54)	4 µM	400 nM	44.5%
PN279	Maleimide-SGRGKQGGKARAKAKTRSSRAGLQFPVGRVHRLLRKG-amide (SEQ ID NO: 55)	40 µM	400 nM	24.7%
PN61	SGRGKQGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGC-amide (SEQ ID NO: 56)	80 µM	800 nM	86.8%
PN360	KGSKKAVTKAQKKDGKKRKRSRK-amide (SEQ ID NO: 57)	80 µM	400 nM	0.0%
PN361	NH2-KKDGKKRKRSRKESYSVYVYKVLKQ-amide (SEQ ID NO: 58)	10 µM	400 nM	42.0%
PN73	KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ (SEQ ID NO: 59)	10 µM	400 nM	99.5%
PN64	BrAc-GWTLNSAGYLLGKINLKALAALAKKILamide (SEQ ID NO: 60)	10 µM	400 nM	14.5%
PN159	KLALKLALKALKAALKLAamide (SEQ ID NO: 13)	.08 µM	80 nM	16.4%
PN68	BrAc-KLALKLALKALKAALKLAamide (SEQ ID NO: 61)	10 µM	400 nM	0.0%
PN182	Ac-KETWWETWWTEWSQPKKKRKV-amide (SEQ ID NO: 62)	1 µM	400 nM	84.9%
PN183	NH2-KETWWETWWTEWSQPGRKKRRQRRRPPQ-amide (SEQ ID NO: 63)	20 µM	400 nM	78.1%

Peptide ID#	Amino Acid Sequence	Peptide Conc.	siRNA Conc.	% Uptake (%PI-/FAM+)
PN71	BrAc-RRRRRRR (SEQ ID NO: 64)	80 µM	400 nM	0.0%
PN87	QqQqQqQqQq (SEQ ID NO: 65)	10 µM	400 nM	0.0%
PN249	NH2-RRRQRRKRGGqQqQqQqQqQ-amide (SEQ ID NO: 66)	80 µM	400 nM	0.0%
PN158	RVIRWFQNKRCKDKK-amide (SEQ ID NO: 67)	1 µM	400 nM	94.0%
PN86	Ac-LGLLLRHLRHHSNLLANI-amide (SEQ ID NO: 68)	80 µM	400 nM	62.2%
PN162	GQMSEIEAKVRTVKLARS-amide (SEQ ID NO: 69)	80 µM	400 nM	0.0%
PN228	NH2-KLWSAWPSLWSSLWKP-amide (SEQ ID NO: 70)	80 µM	400 nM	6.8%
PN357	NH2-KKKKKKKKK-amide (SEQ ID NO: 71)	10 µM	400 nM	0.0%
PN358	NH2-AARLHRFKNKGKDSTEMRRRR-amide (SEQ ID NO: 72)	40 µM	400 nM	0.0%
PN283	Maleimide-GLGSLLKKAGKKLKQPKSKRKV-amide (SEQ ID NO: 73)	40 µM	400 nM	36.3%
PN284	Maleimide-Dmt-r-FK-amide ~~(SEQ ID NO:)~~	100 µM	400 nM	0.0%
PN285	Maleimide-Dmt-r-FKQqQqQqQqQq-amide (SEQ ID NO: 74)	8 µM	800 nM	90.7%
PN286	Maleimide-WRFK-amide (SEQ ID NO: 75)	80 µM	400 nM	0.0%
PN289	Maleimide-WRFKQqQ+qQqQqQq-amide (SEQ ID NO: 76)	8 µM	400 nM	91.7%
PN267	Maleimido-YRFK-amide (SEQ ID NO: 77)	80 µM	400 nM	0.3%
PN282	Maleimide-YRFKYRFKYRFK-amide (SEQ ID NO: 78)	40 µM	800 nM	22.8%
PN286	Maleimide-WRFK-amide (SEQ ID NO: 75)	80 µM	400 nM	0.0%
PN290	Maleimide-WRFKKSKRKV-amide (SEQ ID NO: 79)	80 µM	400 nM	5.3%
PN291	Maleimide-WRFKAAVALLPAVLLALLAP-amide (SEQ ID NO: 80)	4 µM	800 nM	12.5%
PN243	NH2-DiMeYrFKamide (SEQ ID NO: 81)	40 µM	400 nM	0.0%
PN244	NH2-YrFKamide (SEQ ID NO: 82)	80 µM	400 nM	0.0%
PN245	NH2-DiMeYRFKamide (SEQ ID NO: 83)	80 µM	400 nM	0.0%
PN246	NH2-WrFKamide (SEQ ID NO: 84)	80 µM	400 nM	0.0%
PN247	NH2-DiMeYrWKamide (SEQ ID NO: 85)	80 µM	400 nM	0.0%
PN248	NH2-KFrDiMeY-amide (SEQ ID NO: 86)	80 µM	400 nM	0.0%
PN287	Maleimide-WRFKWRFK-amide (SEQ ID NO: 87)	10 µM	400 nM	8.8%
PN288	Maleimide-WRFKWRFKWRFK-amide (SEQ ID NO: 88)	4 µM	400 nM	9.0%

EXAMPLE 4

siRNA/Delivery is Enhanced by Polynucleotide Delivery-Enhancing Polypeptides *In Vitro*

The present example illustrates the enhancement of siRNA uptake by polynucleotide delivery-enhancing polypeptides of the invention in LacZ cells, murine primary fibroblasts and human monocytes. The materials and methods used for the experiments performed in 9L/LacZ cells and mouse fibroblast cells are generally the same as described above, except that for the murine experiments, 9L/LacZ cells were replaced with mouse tail fibroblasts (MTF). The materials and methods used for the experiments performed in human monocytes are described later. The results for transfections performed with MTF cells are summarized in Table 5. Included in Table 5, is the amino acid sequence of the peptide used and the concentration of the peptide and Cy5 label conjugated to the eGFP siRNA. The results for transfections performed

with both MTF and 9L/LacZ cells are summarized in Table 6. The data presented in Table 6 offers a comparison of transfection efficiencies for some peptide/siRNA complexes in different cell types.

Table 5:

Efficiency of siRNA Uptake Mediated by Rationally-Designed Polynucleotide Delivery-Enhancing Polypeptides in Murine Tail Fibroblast (MTF) Cells

Pepdite ID#	Amino Acid Sequence	Status	% Uptake
PN250	NH2-RRRQRRKRGGDIMGEWGNEIFGAIAGFLG-amide (SEQ ID NO: 35)	0.5 mM siRNA/ 40 mM peptide	85.9%
PN73	NH2-KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKV LKQ-amide (SEQ ID NO: 59)	0.5 mM siRNA/ 5 mM peptide	94.5%
PEG-PN509	Peg-KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKV LKQ-amide (SEQ ID NO: 90)	0.5 mM siRNA/ 25 mM peptide	91%
PN404	NH2-RGSRRAVTRAQRRDGRRRRRSRRESYSVYVYRV LRQ-amide (SEQ ID NO: 91)	0.5 mM siRNA/ 25 mM peptide	50.4%
PN361	NH2-KKDGKKRKRSRKESYSVYVYKVLKQ-amide (SEQ ID NO: 58)	0.5 mM siRNA/ 50 mM peptide	65%
PN27	AAVALLPAVLLALLAPRKKRRQRRRPPQC (SEQ ID NO: 38)	0.5 mM siRNA/ 5 mM peptide	60.7%
PN58	NH2-RQIKIWFQNRRMKWKK-amide (SEQ ID NO: 53)	1 mM siRNA/ 20 mM peptide	3.7%
PN158	NH2-RVIRWFQNKRCKDKK amide (SEQ ID NO: 67)	0.5 mM siRNA/ 50 nM peptide	86.2%
PN316	Maleimido-RVIRWFQNKRSKDKK-amide (SEQ ID NO: 92)	0.5 mM siRNA/ 100 mM peptide	84.8%
PN289	Maleimide-WRFKQqQqQqQqQq-amide (SEQ ID NO: 76)	0.5 mM siRNA/ 10 mM peptide	7%
PN28	NH2-RKKRRQRRRPPQCAAVALLPAVLLALLAP-amide (SEQ ID NO: 39)	1 mM siRNA/ 8 mM peptide	80.5%
PN173	GRKKRRQRRRPPQC (SEQ ID NO: 36)	0.5 mM siRNA/ 130 nM peptide	94.8%
PN159	KLALKLALKALKAALKLA-amide (SEQ ID NO: 13)	0.5 mM siRNA/ 5 mM peptide	0%
PN161	NH2-GWTLNSAGYLLGKINLKALAALAKKIL-amide (SEQ ID NO: 93)	0.5 mM siRNA/ 10 nM peptide	0%

Table 6:
Efficiency of siRNA Uptake Mediated by Rationally-Designed
Polynucleotide Delivery-Enhancing Polypeptides in LacZ Cells and Murine Tail Fibroblast Cells

Peptide ID#	Amino Acid Sequence	Percent Uptake	
		LacZ Cells	Primary MTF Cells
PN27	NH2-AAVALLPAVLLALLAPRKKRRQRRRPPQ-amide (SEQ ID NO: 94)	86%	61%
PN28	NH2-RKKRRQRRRPPQAAVALLPAVLLALLAP-amide (SEQ ID NO: 89)	79%	81%
PN29	NH2-AAVALLPAVLLALLAPSGASGLDKRDYV-amide (SEQ ID NO: 47)	87%	not tested
PN58	NH2-RQIKIWFQNRRMKWKK-amide (SEQ ID NO: 53)	76%	6%
PN61	NH2-SGRGKQGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGC-amide (SEQ ID NO: 56)	87%	not tested
PN73	NH2-KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ-amide (SEQ ID NO: 59)	91%	95%
PN158	NH2-RVIRWFQNKRCKDKK-amide (SEQ ID NO: 67)	94%	86%
PN173	NH2-GRKKRRQRRRPPQC-amide (SEQ ID NO: 36)	85%	95%
PN182	NH2-KETWWETWWTEWSQPKKKRKV-amide (SEQ ID NO: 95)	85%	not tested
PN202	NH2-LLETLLKPFQCRICMRNFSTRQARRNHRRRHRR-amide (SEQ ID NO: 50)	71%	not tested
PN204	NH2-C(YGRKKRRQRRRG)2-amide (SEQ ID NO: 42)	83%	not tested
PN250	NH2-RRRQRRKRGGDIMGEWGNEIFGAIAGFLG-amide (SEQ ID NO: 35)	99%	86%
PN361	NH2-KKDGKKRKRSRKESYSVYVYKVLKQ-amide (SEQ ID NO: 58)	42%	65%
PN365	NH2-AAVALLPAVLLALLAPRRRRRR-amide (SEQ ID NO: 45)	81%	not tested
PN404	NH2-RGSRRAVTRAQRRDGRRRRRSRRESYSVYVYRVLRQ-amide (SEQ ID NO: 91)	not tested	50%
PN453	NH2-GALFLGFLGAAGSTMGAWSQPKSKRKVC-amide (SEQ ID NO: 96)	not tested	79%
PN509	Peg-KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ-amide (SEQ ID NO: 90)	not tested	91%

To further characterize the ability of polynucleotide delivery-enhancing polypeptides to transfect cells in culture, human monocytes were incubated with 200 nM of FITC labeled siRNA complexed with various concentrations of PN73, PN250, PN182, PN58 and PN158. Human monocytes were used in addition to LacZ and mouse fibroblast cells because they are the targeted cell type in the treatment of rheumatoid arthritis.

Fresh human blood samples from healthy donors were purchased from Golden West Biologicals. For isolation of monocytes, blood samples were diluted with PBS at a 1:1 ratio immediately after receiving. Peripheral blood mononuclear cells (PBMC) were first isolated by Ficoll (Amersham) gradient from whole blood. Monocytes were further purified from PBMCs using the Miltenyi CD14 positive selection kit and supplied protocol (MILTENYI BIOTEC). To asses the purity of the monocyte preparation, cells were incubated with an anti-CD14 antibody

(BD Biosciences) and then sorted by flow cytometry. The purity of the monocyte preparation was greater than 95%.

The follow description is a brief outline of the transfection protocol used in this example. The cells were plated at 70 ~ 90% confluence for adherent cell lines and 100,000 cells per well for suspension cells. For siRNA/transfection reagent complexes, Cy5- or FAM-conjugated siRNA and peptides were diluted separately in Opti-MEM® media at 2-fold the final concentration. Equal volumes of siRNA and transfection reagent were mixed and allowed to complex 5-10 minutes at room temperature. For siRNA-peptide conjugates, the conjugates were diluted in Opti-MEM® media directly. The transfection mixtures were added to cells previously washed with PBS. Cells were transfected for 3 hours at 37 °C, 5% CO_2. For siRNA uptake analysis, cells were washed with PBS, treated with trypsin (adherent cells only), and then analyzed by flow cytometry. siRNA uptake was measured by the intensity of intracellular Cy5 or FAM fluorescence. Cell viability was determined using propidium iodide (uptake) or AnnexinV-PE (staining).

The forgoing experiment shows that the exemplary polynucleotide delivery-enhancing polypeptide, PN73 is an ideal candidate for the treatment of rheumatoid arthritis. Figure 3 illustrates the ability of several different polynucleotide delivery-enhancing polypeptides to enhance siRNA uptake in human monocytes in culture. Transfection by lipofectamine was used as a comparator. Cell viability was also assessed for each peptide (Figure 4). The data show the surprising and unexpected discovery that the PN73 peptide transfects human monocytes with high efficiency and low toxicity indicating that it is an ideal candidate for the treatment of rheumatoid arthritis *in vivo*.

EXAMPLE 5

siRNA/Delivery is Enhanced by Conjugation of the siRNA to Polynucleotide Delivery-Enhancing Polypeptides

The present example provides results from screens to evaluate activity of siRNA/polynucleotide delivery-enhancing polypeptide conjugates for inducing or enhancing siRNA uptake in 9L/LacZ culture cell lines and primary fibroblast from mouse tail. The materials and methods employed for these studies are generally the same as described above, except that no siRNA/peptide mixing is required as needed to produce siRNA/peptide complexes. The percent uptake for transfections performed with 9L/LacZ cells are summarized in Table 7. Included in Table 7, is the peptide used and the concentration of the peptide/siRNA conjugate. A FAM-β-gal label conjugated to the siRNA molecule was used. The results for transfections performed with MTF are summarized in Table 8. Included in Table 8, is the

concentration of the peptide/siRNA conjugate used and the Cy5 label conjugated to the eGFP siRNA molecule.

Table 7:

Efficiency of siRNA Uptake Mediated by Rationally-Designed Polynucleotide Delivery-Enhancing Polypeptides Conjugated to siRNAs in LacZ Cells

Conjugate Name	Peptide ID#	Peptide/siRNA Conjugate Concentration	Uptake %
CoP267nfR137-1	PN267	tested up to 2.0 μM	0%
CoP286nfR138-1	PN286	0.8 μM	0%
CoP287nfR138-1	PN287	0.8 μM	0%
CoP284nfR164-1	PN284	tested up to 1.0 μM	0%
CoP282nfR165-1	PN282	tested up to 1.0 μM	0%
CoP290nfR165-1	PN290	tested up to 1.0 μM	0%
CoP277nfR167-1	PN73	1.0 μM	42.9%
CoP277nfR167-2	PN73	2.0 μM	55.4%

Table 8:

Efficiency of siRNA Uptake Mediated by Rationally-Designed Polynucleotide Delivery-Enhancing Polypeptides Conjugated to siRNAs in Murine Tail Fibroblast Cells

Conjugate Name	Amino Acid Sequence	Peptide / siRNA Conjugate Concentration	% Uptake
Cy5-dsCoP278nfR270	Maleimide-RRRQRRKRGGDIMGEWGNEIFGAIAGFLG-amide (SEQ ID NO: 102)	0.5μM	96.3%
dsCoP277nfR317	Maleimide-KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ-amide (SEQ ID NO: 103)	4μM	83.5%
dsCoP275nfR321	Maleimide- AAVALLPAVLLALLAPRKKRRQRRRPPQ-amide (SEQ ID NO: 37)	4μM	52.1%
dsCoP285nfR322-1	Maleimide-Dmt-r-FKQqQqQqQqQq-amide (SEQ ID NO: 74)	4uM	41.3%
dsCoP236nfR332	Maleimide-RQIKIWFQNRRMKWKK-amide (SEQ ID NO: 52)	4μM	36.3%
dsCoP317nfR320	Maleimido-KETWWETWWTEWSQPKKKRKV-amide (SEQ ID NO: 104)	2μM	29.6%
dsCoP316nfR347	Maleimido-RVIRWFQNKRSKDKK-amide (SEQ ID NO: 92)	2μM	17.1%
dsCoP289nfR268	Maleimide-WRFKQqQqQqQqQq-amide (SEQ ID NO: 76)	4μM	3.2%
dsCoP276nfR319	Maleimide- RKKRRQRRRPPQCAAVALLPAVLLALLAP-amide (SEQ ID NO: 105)	2μM	3.6%
dsCoP298cfR248	NH2-WRFKC-amide (SEQ ID NO: 106)	4μM	4.1%
dsCoP280nfR362-1	Maleimide-GRKKRRQRRRPPQ-amide (SEQ ID NO: 43)	4μM	1.8%
dsCoP458nfR363-1	Maleimido-KLALKLALKALKAALKLA-amide (SEQ ID NO: 107)	4μM	10.8%
dsCoP459nfR364-1	Maleimido-GWTLNSAGYLLGKINLKALAALAKKIL-amide (SEQ ID NO: 108)	4μM	54.5%

The foregoing data evince that a diverse assemblage of siRNA/peptide conjugates of the invention mediate delivery of siRNAs into different cell types at high efficiency.

EXAMPLE 6

siRNA Gene Expression Knock Down is Enhanced by Polynucleotide Delivery-Enhancing Polypeptides Complexed to siRNA

The instant example demonstrates effective knockdown of target gene expression by siRNA/polynucleotide delivery-enhancing polypeptide complexes of the invention. In the current studies, the ability of peptide/siRNA complexes to modulate expression of a human tumor necrosis factor-α (hTNF-α) gene, implicated as mediating the occurrence or progression of RA when overexpressed in human and other mammalian subjects, was tested.

Healthy human blood was purchased from Golden West Biologicals (CA), the peripheral blood mononuclear cells (PBMC) were purified from the blood using Ficoll-Pague plus (Amersham) gradient. Human monocytes were then purified from the PBMCs fraction using magnetic microbeads from Miltenyi Biotech. Isolated human monocytes were resuspended in IMDM supplemented with 4mM glutamine, 10% FBS, 1x non-essential amino acid and 1x pen-strep, and stored at 4^0C until use.

In a 96 well flat bottom plate, human monocytes were seeded at 100,000 per well per 100 µl in OptiMEM medium (Invitrogen). Transfection reagent was mixed with siRNA at desired concentration in OptiMEM medium at room temperature for 20 minutes (for Lipofectamine 2000; Invitrogen), or 5 minutes (for peptide). At the end of incubation, FBS was added to the mixture (final 3%), and 50 µl of the mixture was added to the cells. The cells were incubated at 37^0C for 3 hours. After transfection, cells were transferred to V-bottom plate, and the cells were pelleted at 1500 rpm for 5 minutes. The cells were resuspended in growth medium (IMDM with glutamine, non-essential amino acid, and pen-strep). After overnight incubation, the cells were stimulated with LPS (Sigma) at 1 ng/ml for 3 hours. After induction, cells were collected as above for mRNA quantification, and supernatant was saved for protein quantification.

For mRNA measurement, branch DNA technology from Genospectra (CA) was used according to manufacturer's specification. To quantitate mRNA level in the cells, both house keeping gene (cypB) and target gene (TNF-α) mRNA were measured, and the reading for TNF-α was normalized with cypB to obtain relative luminescence unit. To quantify protein level, the TNF-α ELISA from BD Bioscience was used according to manufacturer's specification.

The siRNAs directed to target different regions of the TNF-α mRNA as illustrated in Table 9 below. Not shown for each oligo listed in Table 9 is 3' overhangs (e.g. dNdN where N represents any nucleotide).

Table 9:

Nomenclature, Location of Target Sequence in the TNF-α Gene and

Oligo Sequence for siRNA Targeting of TNF-α

ID#	siRNA Name	Location of Target sequence	Oligo Sequence	SEQ ID NO:
N125	TNF-α-1	516-534	GCGUGGAGCUGAGAGAUAA	109
N115	TNF-α-2	430-448	GCCUGUAGCCCAUGUUGUA	110
N132	TNF-α-3	738-756	GGUAUGAGCCCAUCUAUCU	111
N108	TNF-α-4	360-378	CCAGGGACCUCUCUCUAAU	112
N138	TNF-α-5	811-829	GCCCGACUAUCUCGACUUU	113
N113	TNF-α-6	424-442	UGACAAGCCUGUAGCCCAU	114
N143	TNF-α-7	844-862	GGUCUACUUUGGGAUCAUU	115
N107	TNF-α-8	359-377	CCCAGGGACCUCUCUCUAA	116
N137	LC1	806-828	AAUCGGCCCGACUAUCUCGACUU	117
N122	LC2	514-532	AAUGGCGUGGAGCUGAGAGAU	118
N130	LC3	673-691	AACCUCCUCUCUGCCAUCAAG	119
N101	LC4	177-195	AACUGAAAGCAUGAUCCGGGA	120
N140	LC5	820-838	AAUCUCGACUUUGCCGAGUCU	121
N135	LC6	781-799	AAGGGUGACCGACUCAGCGCU	122
N128	LC7	636-654	AAUCAGCCGCAUCGCCGUCUC	123
N127	LC8	612-630	AACCCAUGUGCUCCUCACCCA	124
N118	LC9	472-490	AAGCUCCAGUGGCUGAACCGC	125
N111	LC10	398-416	AAGUCAGAUCAUCUUCUCGAA	126
N110	LC11	363-381	AAGGGACCUCUCUCUAAUCAG	127
N105	LC12	265-287	CCUCAGCCUCUUCUCCUUCCUGA	128
N104	LC13	264-282	AAUCCUCAGCCUCUUCUCCUU	129
N120	LC14	495-513	AACCAAUGCCCUCCUGGCCAA	130
N153	LC16	1535-1555	CUGAUUAAGUUGUCUAAACAA	131
N136	LC17	787-807	CCGACUCAGCGCUGAGAUCAA	132
N152	LC18	1327-1347	CUUGUGAUUAUUUAUUAUUUA	133
N114	LC19	428-448	AAGCCUGUAGCCCAUGUUGUA	134
N145	LC20	982-1002	UAGGGUCGGAACCCAAGCUUA	135
N101	YC-1	177-195	CUGAAAGCAUGAUCCGGGA	136
N103	YC-2	251-269	AGGCGGUGCUUGUUCCUCA	137
N106	YC-3	300-318	CCACCACGCUCUUCUGCCU	138
N109	YC-4	362-380	AGGGACCUCUCUCUAAUCA	139
N113	YC-5	424-442	UGACAAGCCUGUAGCCCAU	140
N115	YC-6	430-448	GCCUGUAGCCCAUGUUGUA	141
N117	YC-7	435-453	UAGCCCAUGUUGUAGCAAA	142
N120	YC-8	495-513	CCAAUGCCCUCCUGGCCAA	143
N121	YC-9	510-528	CCAAUGGCGUGGAGCUGAG	144
N123	YC-10	515-533	GGCGUGGAGCUGAGAGAUA	145
N125	YC-11	516-534	GCGUGGAGCUGAGAGAUAA	146
N126	YC-12	558-576	GCCUGUACCUCAUCUACUC	147
N130	YC-13	673-691	CCUCCUCUCUGCCAUCAAG	148
N132	YC-14	738-756	GGUAUGAGCCCAUCUAUCU	149
N133	YC-15	772-790	GCUGGAGAAGGGUGACCGA	150
N134	YC-16	776-794	GAGAAGGGUGACCGACUCA	151
N136	YC-17	787-807	GCCCGACUAUCUCGACUUU	152
N141	YC-18	841-859	GCAGGUCUACUUUGGGAUC	153
N143	YC-19	844-862	GGUCUACUUUGGGAUCAUU	154
N144	YC-20	853-871	UGGGAUCAUUGCCCUGUGA	155

ID#	siRNA Name	Location of Target sequence	Oligo Sequence	SEQ ID NO:
N146	YC-21	985-1003	GGUCGGAACCCAAGCUUAG	156
N147	YC-22	1179-1197	CCAGAAUGCUGCAGGACUU	157
N148	YC-23	1198-1216	GAGAAGACCUCACCUAGAA	158
N149	YC-24	1200-1218	GAAGACCUCACCUAGAAAU	159
N150	YC-25	1250-1268	CCAGAUGUUUCCAGACUUC	160
N151	YC-26	1312-1330	CUAUUUAUGUUUGCACUUG	161
N154	YC-27	1547-1565	UCUAAACAAUGCUGAUUUG	162
N155	YC-28	1568-1585	GACCAACUGUCACUCAUU	163

Tables 10, 11 and 12 illustrate the effectiveness of specific oligos complexed to a polynucleotide delivery-enhancing polypeptides of the invention to target and knock down TNF-α gene expression levels in human monocytes.

Table 10:

Percent TNF-α Knockdown Mediated by a PN73/siRNA Complex

Peptide/siRNA Complex		
	siRNA (4 nM)	**KD (%)**
Peptide PN73 (1.6 μM)	LC1	20.08%
	LC2	19.06%
	LC3	23.17%
	LC4	26.67%
	LC5	46.78%
	LC6	44.10%
	LC7	42.76%
	LC8	41.24%
	LC9	40.32%
	LC10	13.52%
	LC11	7.89%
	LC12	40.61%
	LC13	48.29%
	LC14	50.76%
	LC16	55.91%
	LC17	50.78%
	LC18	63.44%
	LC19	61.83%
	LC20	42.68%
	YC12	43.60%

Table 11:

Percent TNF-α Knockdown Mediated by a PN509/siRNA Complex

	Peptide/siRNA Complex		
		siRNA (4 nM)	**KD (%)**
Target Gene TNF-α	**Peptide PN509 (1.6 μM)**	LC1	31.13%
		LC2	37.04%
		LC3	30.14%
		LC4	22.71%
		LC5	34.93%
		LC6	50.19%
		LC7	56.11%
		LC8	47.35%
		LC9	58.20%
		LC10	25.62%
		LC11	25.65%
		LC12	17.03%
		LC13	25.04%
		LC14	42.78%
		LC16	40.06%
		LC17	48.94%
		LC18	58.13%
		LC19	56.38%
		LC20	71.12%
		YC12	64.37%

Table 12:

Percent TNF-α Knockdown Mediated by a PN250/siRNA Complex

	Complex		
	Peptide PN250 Conc.	**siRNA 20nM**	**KD (%)**
Target Gene TNF-α	**0.5 μM**	YC11	13.70%
		YC12	17.06%
		YC17	17.30%
		YC18	20.72%
		LC13	20.65%
		LC20	-3.80%
		TNF-4	0.90%
	0.75 μM	YC11	21.09%
		YC12	21.66%
		YC17	29.82%
		YC18	17.82%
		LC13	18.04%
		LC20	10.72%
		TNF-4	14.39%
	1 μM	YC11	33.10%
		YC12	15.91%
		YC17	24.68%
		YC18	24.66%
		LC13	31.35%
		LC20	26.53%
		TNF-4	26.47%

The foregoing results (Tables 10, 11 and 12) evince that effective levels of TNF-α gene expression knock down can be achieved in mammalian cells using the novel siRNA/polynucleotide delivery-enhancing polypeptide compositions of the invention.

Screening and Characterization

Human monocytes (CD14+) treated with LPS induce TNF-α-specific mRNA within approximately 2 hours, followed by peak levels of TNF-α protein 2 hours later. siRNAs were screened for knockdown activity by transfecting monocytes with siRNA candidate sequences using Lipofectamine 2000, treating infected cells with LPS, and measuring TNF-α mRNA levels approximately 16 hrs later. Fifty six siRNA sequences were designed and screened for their ability to knockdown TNF-α mRNA and protein levels in activated human primary monocytes. Activities for a representative set of 27 siRNA sequences ranged from 80% mRNA knockdown activity to no detectable activity. In general, TNF-α protein levels were reduced more than mRNA levels, e.g., a 50% knockdown in TNF-α mRNA (TNF-α-1) resulted in a 75% reduction in TNF-α protein level. Dose response curves for selected siRNAs that exhibited knockdown levels from 30% to 60 % were obtained. Calculated IC_{50}values were in the 10 pMolar to 200 pMolar range. While the siRNA sequences evaluated were distributed throughout the TNF-α transcript, the most potent siRNAs identified were located in two areas: the middle of the coding region and the 3'-UTR.

EXAMPLE 7

siRNA Gene Expression Knock Down is Enhanced by Polynucleotide Delivery-Enhancing Polypeptides Conjugated with siRNA

The present example demonstrates knockdown of target gene expression by peptide-siRNA conjugates of the invention. The materials and methods for these studies are the same as those described above, with the exception that no mixing of the siRNA and peptide is required. In the present series of studies, the knockdown experiments included comparison of peptide/siRNA-mediated knockdown with and without lipofectamine. The results of this example are illustrated in Table 13 below.

Table 13:

Peptide/siRNA-Mediated Knockdown of

TNF-α Gene Expression With and Without Lipofectamine

Cell Type Used in Assay	Complex Name	Peptide + siRNA	With Lipofectamine		Without Lipofectamine	
			Conc. (μM)	KD (%)	Conc. (μM)	KD (%)
CD14	CoP456	cIBR + LC20	0.4 μM	no KD	0.4 μM	no KD
			1.3 μM	no KD	1.3 μM	no KD
			4 μM	no KD	4 μM	no KD
	CoP457	Peptide T + LC20	0.4 μM	no KD	0.4 μM	no KD
			1.3 μM	no KD	1.3 μM	no KD
			4 μM	no KD	4 μM	no KD
	CoP278	TAT/HA + YC12	0.4 μM	no KD	0.4 μM	no KD
			1.3 μM	no KD	1.3 μM	no KD
			4 μM	no KD	4 μM	no KD
MTF	CoP277	PN73 + LC13	0.19 μM	31.95%	0.19 μM	61.61%
			0.38 μM	32.83%	0.38 μM	76.31%
			0.75 μM	39.29%	0.75 μM	73.94%
			1.50 μM	41.42%	1.50 μM	73.14%
			3.00 μM	39.88%	3.00 μM	58.14%
			6.00 μM	20.23%	6.00 μM	50.71%
CD14	CoP277	PN73 + LC13			0.000 μM	93.06%
					0.002 μM	83.63%
					0.011 μM	72.58%
					0.053 μM	73.52%
					0.266 μM	85.01%
CD14	CoP277	PN73 + LC20			0.000 μM	75.15%
					0.002 μM	60.72%
					0.011 μM	57.09%
					0.053 μM	58.70%
					0.266 μM	62.79%

The foregoing data (Table 13) evince that a diverse assemblage of polynucleotide delivery-enhancing polypeptides of the invention conjugated with siRNAs function to enhance siRNA-mediated knockdown of TNF-α gene expression in mammalian subjects.

EXAMPLE 8

Time Course of siRNA Gene Expression Knock Down

The instant example presents studies relating to the time course of siRNA-mediated gene expression knockdown. To test the duration of the siRNA effect, the siRNA transfection procedures as noted above were employed, except that fibroblasts derived from eGFP expressing mice were used. The transfection reagent used here was lipofectamine. The cells were replated

on the 18th day due to overgrowth. The second transfection was performed on the 19th day post first transfection. On the 19th day the eGFP levels were measured after the transfection. Scramble or nonsense siRNA (Qiagen) was used as a control, along with a GFPI siRNA (GFPI) and a hairpin siRNA (D#21). The knockdown activities were calibrated with scramble siRNA (Qiagen control). A higher value indicates greater knockdown activity.

Table 14:

Time Course of TNF-α Gene Expression

Knock Down by Lipofectamine Mediated Transfection of siRNA

siRNA	Days Post First Transfection								
	1	3	5	7	9	11	13	15	17
Scramble siRNA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
GFPI	27.61	60.87	64.75	58.40	56.72	40.46	35.56	16.59	15.50
D#21	28.22	61.11	66.91	62.86	57.36	54.71	42.96	24.66	9.88

siRNA	Days Post First Transfection				
	19	20	21	25	27
Scramble siRNA	0.00	0.00	0.00	0.00	0.00
GFPI	59.60	37.10	57.38	66.94	59.63
D#21	46.36	35.89	65.25	74.15	58.39

The foregoing studies (Table 14) demonstrate that siRNA knockdown activity became apparent around day 3, and was sustained through day 9, whereafter target gene expression returned to baseline levels around day 17. After the second transfection on day 18, another reduction of eGFP expression occurred indicating that the reagent can be repeatedly administered to cells to yield repeated or enduring gene expression knockdown.

EXAMPLE 9

Dosage Dependence of TNF-α Gene Expression Knock Down Mediated by siRNA Complexed With Polynucleotide Delivery-Enhancing Polypeptide

The present example demonstrates that knockdown activity mediated by siRNA complexed with an exemplary polynucleotide delivery-enhancing polypeptide, PN73, in activated human monocytes is dependent on dosage concentration of peptide-siRNA complex.

The PN73/siRNA complex was provided in a constant ratio between PN73 and siRNA of about PN73:siRNA=82:1 (Table 15). Four hundred nanomolar siRNA was complexed with 33μM PN73 for 5 minutes in OptiMEM medium. After complexation, the complex were serial diluted (1:2 ratio) with OptiMEM. The complex was added to human monocytes for

transfection. The following induction and mRNA quantification was performed according to the description above.

Table 15:

Peptide Dosage Dependence of TNF-α Gene Expression Knock Down

PN73 Conc. (μM)	siRNA Conc. (nM)	Gene expression (%)			
		Control	TNF-α2	TNF-α4	LC8
0 μM	0 nM	100%	100%	100%	100%
1.2 μM	14.81 nM	99.99%	80.28%	70.22%	73.44%
3.6 μM	44.44 nM	100.11%	69.33%	62.97%	63.04%
11 μM	133.33 nM	99.99%	57.82%	62.71%	59.57%
33 μM	400.00 nM	99.99%	64.51%	78.48%	51.30%

In a related series of experiments, siRNA was serially diluted and combined with a fixed amount of PN73 (1.67 μM). Alternatively stated, the PN73 polynucleotide delivery-enhancing polypeptide was complexed with titration amounts of siRNA. PN73 (1.67 μM) was complexed with each titration amount of LC20 siRNA for 5 minutes at room temperature in OptiMEM medium. After complexation, the complex was added to human monocytes for transfection. The induction and mRNA quantification data provided in Table 16, below, were obtained by methods described above.

Table 16:

siRNA Dosage Dependence of TNF-α Gene Expression Knock Down

siRNA conc. (nM)	Control (%)	TNF-α Gene expression (%)
0.8 nM	100.0%	84.7%
4 nM	100.0%	59.4%
20 nM	100.0%	65.2%
100 nM	100.0%	54.7%

EXAMPLE 10

Multiple Dosing Protocol to Extend siRNA Knockdown Effect in Mammalian Cells

The instant example demonstrates that multiple dosing schedules will effectively extend gene expression knockdown effects in mammalian cells mediated by siRNA/polynucleotide delivery-enhancing polypeptide compositions of the invention. The materials and methods employed for these studies are the same as described above, with the exception that repeated transfections were conducted at the times indicated. The scramble siRNA (Qiagen) was utilized

for side by side controls. Table 17 summarizes the data for multiple transfections with a peptide/siRNA complex. The percent knockdown activity of the TNF-α gene represents the percent of total gene expression.

Table 17:

TNF-α Gene Expression Knock Down Activity

After Multiple Transfections With a Peptide/siRNA Complex

Dosing Regime	Days post 1st Transfection								
	4	5	6	7	8	9	10	11	12
Single	74.7%	61.9%	62.6%	55.5%	41.4%	39.4%	27.2%		
2nd on 5th			66.7%	69.8%	68.3%	64.2%	63.9%	64.4%	56.5%
2nd on 6th				64.2%	65.8%	67.7%	64.1%	58.6%	54.0%
2nd on 7th					63.1%	62.5%	69.9%	62.6%	58.1%

The foregoing results (Table 17) demonstrate that when multiple transfections are performed timely (in this case between about the 5th-7th day post first transfection), TNF-α gene expression knockdown effects in mammalian cells can be maintained or re-induced.

EXAMPLE 11

In Vivo siRNA/Peptide-Mediated TNF-α Gene Expression Knock Down Activity

The present example provides *in vivo* data demonstrating the efficacy of siRNA/polynucleotide delivery-enhancing polypeptide compositions of the invention to mediate systemic delivery and therapeutic gene knockdown by siRNA, effective to modulate target gene expression and modify phenotype of cells in a therapeutic manner.

Human TNF-α expressing mice were purchase from the Hellenic Pasture Institute, Greece) at 5weeks old. Mice were administered through intra venous (IV) with 300 μl saline twice a week (4 mice), with the RA drug Ramicade (5 mg/kg) once a week (2 mice), or with LC20 siRNA (2 mg/kg) mixed with PN73 at 1:5 molar ratio twice a week (2 mice). During the injection periods, plasma samples were collected for ELISA testing (R&D Systems), and paw scores were taken twice a week as an accepted index of RA disease progression and therapeutic efficacy. TNF-α protein blood plasma levels from treated mice are shown below in Table 18.

Table 18:

Quantity of TNF-α Protein in Blood Plasma as Assayed by ELISA

Treatment	Age(week)		
	7	8	9
Ramicade	102.24	39.27	25.80
LC20/PN73	25.96	21.89	14.21
Saline	33.78	34.29	24.48

**These data represent the average of the mice in the experiment in pg/ml.*

The foregoing results demonstrate effective reduction of TNF-α protein levels in peptide/siRNA-treated mice in the circulating blood as compared to levels in Ramicade or saline (control) treated mice.

Additional evidence of *in vivo* efficacy of the siRNA/polynucleotide delivery-enhancing polypeptide compositions and methods of the invention were obtained from the above murine subjects using paw scores, an accepted phenotypic index for RA disease status and treatment efficacy. Due to the difference in the starting point (some animals present with scores at earlier points), the scores have been adjusted to 0 for all animals in the experiments. Each paw is given a score between 0 and 3, with the highest score of 12, according to the following scoring index.

0: Normal

1: edema or distortion of paw or ankle joints

2: distortion of paw and ankle joints

3: ankylosis of wrist or ankle joints.

The results of these paw score evaluations are presented graphically in Figure 5. The data demonstrate that the polynucleotide delivery-enhancing polypeptide PN73 can deliver therapeutic amounts of siRNAs (e.g., LC20, TNF-α2, and TNF-α9 (UAGCCCAUGUUGUAGCAAA (SEQ ID NO. 187))) when injected into animals as shown by a delayed RA progression at week 8. The PN73/siRNA treated mice faired better on the paw scoring index at week 8 compared to the Ramicade-treated mice. When paw score evaluations were carried out to 11 weeks post-treatment, PN73/LC20 complex achieved comparable paw score evaluations to the Ramicade-treated mice. At a ratio of 1:5 for the PN73 peptide/LC20 siRNA, 2 mg/kg LC20 achieved the greatest relative observed delay in RA progression compared to the lower doses of LC20 tested. The Table 19 below summarizes the relative effectiveness of several siRNAs for 5 different groups evaluated after treatment with PN73 and siRNAs.

Table 19:
Group Summaries

Group Label	Treatment*	Relative Effect of siRNA
TNF #1	LC20, Ramicade, PBS	LC20 is as effective as Ramicade
TNF #4	LC20 and LC13	Overall low paw score
TNF #5	LC20 conjugated to PN73	Overall low paw score; Conjugates have lower activity than Complexes
TNF #6	LC20, YC12 and LC17	Overall low paw score. YC12 and LC17 not as effective as LC20
TNF #7 (Figure 5)	LC20, TNF-α2 and TNF-α9	LC20 and TNF-α9 are more effective than Ramicade by week 8; LC20 is equally effective as Ramicade by week 11

siRNAs were tested in the presence of absence of PN73; Ramicade is a positive treatment control; PBS is a negative treatment control.

The foregoing results demonstrate that siRNA and polynucleotide delivery-enhancing polypeptide compositions of the invention provide promising new therapeutic tools for regulating gene expression and treating and managing disease. siRNAs of the invention, for example siRNAs targeting human TNF-α-specific mRNAs for degradation, offer higher specificity, lower immunogenicity and greater disease modification than current small molecule, soluble receptor, or antibody therapies for RA. More than 50 candidate siRNA sequences were screened that targeted hTNF-α and yielded single administration knockdowns of 30 to 85%. Over 20 *in silico* designed peptide complex and/or covalent molecules were compared for fluorescent RNA uptake by monocytes and a number were found to have significantly better uptake than Lipofectamine or cholesterol-conjugated siRNA and with < 10 pM IC_{50} values. The peptide-siRNA formulations efficiently knockdown TNF-α mRNA and protein levels in activated human monocytes *in vitro*.

One exemplary candidate delivery peptide/siRNA formulation was evaluated in two transgenic mouse models of rheumatoid arthritis (RA) constitutively expressing human TNF-α. Animals treated with 2 mg/kg siRNA by IV injection or infliximab twice weekly beginning at age 6 weeks showed RA score stabilization (paw and joint inflammation) beginning at age 7 weeks, compared to controls where these disease conditions persisted through week 10. At age 9 weeks, siRNA treated animals showed comparable reductions in RA scores, but significantly lower plasma TNF-α protein levels than infliximab treated animals.

Based on the disclosure herein, the use of siRNA to inhibit the expression of target genes, for example cytokines such as TNF-α, that play important roles in pathological states, such as

inflammation, provides effective treatments to alleviate or prevent symptoms of disease, as exemplified by RA, in mammalian subjects. Exemplary peptide/siRNA compositions employed within the methods and compositions of the invention provide advantages relating to their ability to reduce or eliminate target gene expression, e.g., TNF-α expression, rather than by complexing with the product of the target gene, e.g., TNF-α, as in the case of antibodies or soluble receptors.

Improving systemic delivery of nucleic acids according to the teachings of the invention provides yet additional advantages for development of siRNAs as drugs. Specific challenges in this context include delivery through tissue barriers to a target cell or tissue, maintaining the stability of the siRNA, and intracellular delivery getting siRNAs across cell membranes into cells in sufficient quantities to be effective. The present disclosure demonstrates for the first time an effective *in vivo* delivery system comprising novel peptide/siRNA compositions targeting specific gene expression, such as expression of human TNF-α, which attenuate disease activity in transgenic animal models predictive of target diseases, as exemplified by studies using murine models of RA. In related studies, the compositions and methods of the invention effectively inhibit TNF-α expression in activated monocytes derived from patients with RA. These results indicate that the RNAi pathway effectively mediates alteration of cellular phenotype and disease progression through intracellular effects on TNF-pathways, and avoids toxicity effects due to circulating antibody/TNF-α complexes with residual immunoreactivity that characterize current antibody therapies for RA. Notably, all of the tests herein were implemented with associated toxicity effects minimized, such that the dosages of siNAs and polynucleotide delivery-enhancing polypeptides shown in these examples always correlated with cell viability levels of at least 80-90% or greater.

EXAMPLE 12

Optimizing Rational Design of Polynucleotide Delivery-Enhancing Polypeptides

The instant example provides an exemplary design and data for optimizing polynucleotide delivery-enhancing polypeptides of the invention. The subject rational design manipulations were conducted for a histone H2B-derived polynucleotide delivery-enhancing polypeptide.

Table 20:

Deletion and Modification of PN73

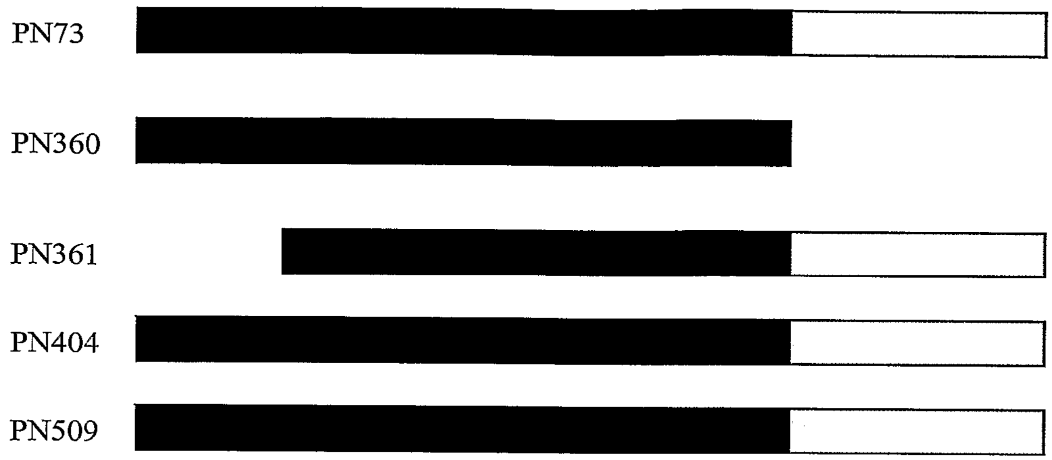


Table 20 provides a diagram of the primary structure of PN73 and its derivatives generated for optimizing rational design of PN73-based polynucleotide delivery-enhancing polypeptides. The gray colored C-terminus of each peptide represents the hydrophobic domain of the peptide and the black N-terminus of each peptide represents the hydrophilic domain. The parent peptide PN73 was demonstrated above to be an example of a polynucleotide delivery-enhancing polypeptides for inducing or enhancing siRNA delivery to cells.

In order to better understand the function-structural activity relationships of this and other polynucleotide delivery-enhancing polypeptides, primary structural studies were performed by characterizing C- and N-terminal function, and activity of conjugates between PN73 and other chemical moieties.

The amino acid sequence for the human histone 2B (H2B) protein is shown below.

PN73, PN360 and PN361 are peptide fragments of H2B and the portion of the H2B protein that they represent are identified below in parentheses following the peptide name. The amino acids sequence for PN360 and PN361 listed below are aligned with the corresponding amino acid sequence found in PN73. The PN73 peptide fragment is underlined in the H2B amino acid sequence and represents H2B amino acids 13 through 48. It may also be represented by H2B amino acids 12 through 48. PN360 shares the N-terminus with PN73 but lacks PN73's C-terminus while PN361 shares the C-terminus with PN73 but lacks PN73's N-terminus. The PN73 conjugate is PN73 covalently linked to a single siRNA strand (e.g., sense strand). PN404

is a version of PN73 in which all of lysines are replaced with arginines and PN509 is a pegylated PN73 (PEG molecular weight 1k Dalton) derivative that is pegylated at the N-terminus.

H2B (histone 2B) amino acid sequence

MPEPAKSAPAPKKGSKKAVTKAQKKDSKKRKRSRKESYSVYVYKVLKV
HPDTGISSKAMGIMNSFVNDIFERIAGEASRLAHYNKRSTITSREIQTAVRL
LLPGELAKHAVSEGTKAVTKYTSSK (SEQ ID NO: 164)

PN73 (13-48)

NH2-KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ-amide (SEQ ID NO: 59)

PN360 (13-35; N-terminus of PN73)

NH2-KGSKKAVTKAQKKDGKKRKRSRK-amide (SEQ ID NO: 57)

PN361 (24-48; C-terminus of PN73)

NH2-KKDGKKRKRSRKESYSVYVYKVLKQ-amide (SEQ ID NO: 58)

PN73 (13-48)-siRNA (sense strand) conjugates

siRNA-KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ-amide (SEQ ID NO 59)

PN404 (PN73 where all lysines are replaced with arginines)

NH2-RGSRRAVTRAQRRDGRRRRRSRRESYSVYVYRVLRQ-amide (SEQ ID NO: 91)

PN509 (pegylated PN73)

PEG- RGSRRAVTRAQRRDGRRRRRSRRESYSVYVYRVLRQ-amide (SEQ ID NO: 90).

Figure 6 provides the results of uptake efficacy and viability studies in mouse tail fibroblast cells for the foregoing PN73 rationally-designed derivative polynucleotide delivery-enhancing polypeptides. The activity changes of modified PN73 in mouse tail fibroblast cells are illustrated. Unlike PN404, PN509 increases uptake without increasing toxicity. While deleting part of the N-terminus of PN73 reduces activity, removal of C-terminal residues

abolishes the activity. Both PN73 and PN509 show higher activity in primary cells than Lipofectamine (Invitrogen, CA).

EXAMPLE 13

Acetylated Polynucleotide Delivery-Enhancing Polypeptide Has Increased Stability in Plasma

The purpose of the instant example was to determine if modification of the exemplary polynucleotide delivery-enhancing polypeptide PN73 would provide increased stability to the peptide and consequently enhance its transfection activity. The stability of unmodified, N-terminus pegylated and N-terminus acetylated forms of PN73 in plasma was compared. The C-terminus of the PN73 is amidated. Size exclusion chromatography coupled with an ultraviolet detector were used to characterize the stability of the unmodified and modified forms of PN73 before and after incubation in plasma.

In the absence of plasma, the unmodified, pegylated and acetylated forms of PN73 showed distinct yet overlapping UV traces at approximately 9 minutes. However, after 4 hours of exposure to plasma, UV traces specific to unmodified PN73 and pegylated PN73 were no longer present indicating significant degradation. In contrast, the distinct UV trace for acetylated PN73 remained indicating that this modification significantly increased stability of the PN73 in plasmid compared to the unmodified and pegylated PN73 forms.

These data show the surprisingly and unexpected discovery that PN73 stability in plasma can be enhanced by N-terminus acetylation of the PN73 peptide. The primary structure of the acetylated PN73 peptide is as follows:

Ac-KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ-amide (SEQ ID NO: 59)

EXAMPLE 14

Polynucleotide Delivery-Enhancing Polypeptide Does Not Elicit an Interferon Response

The purpose of the instant example was to compare the interferon response of cells transfected with either liposomal reagent plus siRNA or the exemplary polynucleotide delivery-enhancing polypeptide, PN73 peptide plus siRNA. Interferon responsiveness was assayed by ELISA (protein) and bDNA (mRNA levels).

Traditionally, siRNA molecules are delivered into cells by a liposomal mediated transfection. However, this typically results in a poor efficiency of delivery, an inflammatory response *in vivo* and an upregulation of interferon gene expression which results in an inhibition of cell growth. Consequently, there is a limited reduction in targeted gene expression levels thus making siRNA an ineffective method of treatment and tool for studying gene expression. Delivery of siRNA by PN73 overcomes this problem.

Figure 7 provides the results of the bDNA assay of lipofectamine versus PN73 peptide transfection of several different siRNAs. siRNAs were complexed with either lipofectamine or PN73 at concentrations of 1 nM, 10 nM, 100 nM or 200 nM. Interleukin 1β (IL-1β) served as a molecular marker to determine interferon responsiveness and Qneg was used as a negative control. As shown in Figure 7, lipofectamine complexed with the 100 nM or 200 nM TNF-α9 siRNA caused a significant increase in IL-1β mRNA levels. Furthermore, all other siRNAs tested caused a mild increase in IL-1β mRNA levels. In contrast, the same siRNAs complexed with the PN73 peptide did not cause an increase in IL-1β mRNA levels.

To further characterize the difference in interferon responsiveness observed between cells transfected with either lipofectamine and PN73 transfection, an ELISA assay was performed to determine the protein expression levels of the following molecular markers: Interleukin 1β (IL-1β), Interferon-α (INF-α), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-12 (IL-12), MIP-1α, Interferon-γ (IFN-γ), and Tumor Necrosis Factor-α (TNF-α). Table 21 summarizes the relative protein expression levels of cells transfected with Lipofectamine complexed with siRNA or PN73 complexed with siRNA.

Table 21:

Relative Protein Expression Levels of Molecular Markers of Interferon Responsiveness

	Lipofectamine Transfection				**PN73 Transfection**
Interferon Response Marker	**IFN-1 siRNA**	**LC17 siRNA**	**LC20 siRNA**	**TNF-α9 siRNA**	**For All siRNAs (IFN-1; LC17; LC20; TNF-α9)**
IL-1β	++	-	-	+	-
INF-α	Background	Background	Background	Background	Background
IL-6	++	-	-	+	-
IL-8	-	-	-	-	-
IL-12	Background	Background	Background	Background	Background
MIP-1α	+++	-	-	++	-
IFN-γ	Background	Background	Background	Background	Background
TNF-α	+	-	-	-	-

As presented in Table 21, both siRNA LC20 and LC17 had no interferon response regardless of what transfection reagent was used. However, transfection of IFN-1 or TNF-α9 with lipofectamine caused an increase in IL-1β, IL-6, and MIP-1α, protein expression levels. In contrast, transfection of all tested siRNAs with PN73 caused no observable induction in protein expression in any of the interferon response markers tested.

These data from the ELISA assay show the surprisingly and unexpected discovery that PN73 mediated transfection of siRNAs does not elicit an interferon response.

EXAMPLE 15

siRNA Conjugated with a Polynucleotide Delivery-Enhancing Polypeptide Provides Greater Knockdown Activity than siRNA Complexed with Polynucleotide Delivery-Enhancing Polypeptide

The purpose of the instant example was to compare the knockdown activities in human monocytes of the siRNAs LC13 and LC20 either conjugated or complexed with the exemplary polynucleotide delivery-enhancing polypeptide PN73. Isolation and transfection of human monocytes as well as the methods used to measure knockdown activity were discussed earlier. Qneg represents a random siRNA sequence and functions as the negative control in these experiments. The observed Qneg knockdown activity is normalized to 100% (100% gene expression levels) and the activity of LC20 and LC13 is presented as a relative percentage of the negative control. LC20 and LC13 are siRNAs targeted against the human TNF–α gene. Figure 8 shows the knockdown activity for the siRNAs LC20 and LC13 without PN73 (Figure 8-C), complexed with PN73 (Figure 8-B) or conjugated with PN73 (Figure 8-A). LC20 and L13 were tested over a concentration range of 0 nM to 2.5 nM. PN73 was kept at a 1:1 ratio in both the complex and conjugate experiments.

As expected, in the absence of PN73, LC13 and L20 showed little knockdown activity (Figure 8-C). Both LC13 and LC20 caused an approximate 15% and 30% decrease in TNF-α gene expression relative to the Qneg control when complexed with PN73 (Figure 8-B). However, knockdown activity for TNF-α was reduced to below 60% when the siRNA was conjugated to PN73 (Figure 8-A). This is significant increase the siRNA knockdown activity compared to the PN73/siRNA complex. Thus, these data show the surprisingly and unexpected discovery that siRNA knockdown activity is significantly enhanced when the siRNA is conjugated to the exemplary polynucleotide delivery-enhancing polypeptide PN73.

EXAMPLE 16

Serum Inhibition of Cholesterol-Enhanced siRNA Uptake is Rescued by a Polynucleotide Delivery-Enhancing Polypeptide

The present example demonstrates that the addition of permeabilizing peptide to a delivery formulation comprising a siRNA conjugated to a cholesterol moiety reduces the inhibitory effects of serum on cholesterol-siRNA uptake in a dose dependent manner. For siRNA uptake analysis, cells were washed with PBS, treated with trypsin (attached cells only), and then analyzed by flow cytometry. Uptake of the siRNA designated BA, described above, was also measured by intensity of Cy5 or FAM fluorescence in the cells and cellular viability assessed by addition of propidium iodide or AnnexinV-PE. In order to differentiate the cellular

uptake from the membrane insertion of fluorescence labeled siRNA, trypan blue was used to quench the fluorescence on the cell membrane surface.

Table 22:

PN73 Mediated Transfection Rescues Serum Induced Inhibition of Cholesterol Conjugated siRNA Cell Uptake as Assayed by Mean Fluorescent Intensity (MFI)

Percent Serum	Cholesterol siRNA alone (MFI)	Unconjugated siRNA with 20 μM PN73 (MFI)
0	24.8	32.9
5%	1.55	11.5
10%	1.34	6.39
20%	1.19	5.85

The data in Table 22 shows that the presence of serum significantly reduces cellular uptake of the siRNA conjugated to a cholesterol moiety according to the invention. However, unconjugated siRNA cellular uptake is rescued in the presence of an exemplary delivery-enhancing peptide, PN73.

A comparison of cellular uptake of a cholesterol-conjugated siRNA according to the invention in complex with a permeabilizing peptide delivery enhancing agent, PN73 (cholesterol siRNA+PN73), and on an unconjugated siRNA in complex with PN73 (siRNA+PN73) was performed. As shown in Figure 9, equivalent cell uptake activity can be achieved at high concentrations of PN73 with either unconjugated siRNA or a cholesterol-conjugated siRNA. For these and related uptake assays, cholesterol-conjugated siRNA and siRNA/PN73 complex were transfected into mouse tail fibroblast cells (MTF) in Opti-MEM® media (Invitrogen) as described above, with serum added in fixed or varied concentration(s). The final concentration of siRNA for both cholesterol and complex were 0.2 μM. The uptake efficiency and Mean fluorescence intensity were assessed by flow cytometry.

The ability of the PN73 and additionally PN250 to rescue serum inhibition of cell uptake of cholesterol conjugated siRNA was further characterized in mouse tail fibroblast (MTF) cells. Figure 10 illustrates the effect of increasing concentration of fetal bovine serum (FBS) on siRNA cell uptake. In the absence of either PN73 or PN250, the cell uptake of cholesterol conjugated siRNA is drastically compromised at just 5% FBS. However, in the presence of either 40 μM PN73 or 80 μM PN250, siRNA uptake is rescued.

The foregoing data (Table 22 and Figure 10) demonstrate that cholesterol-conjugation of siRNAs can significantly enhance their cellular uptake. However, uptake of cholesterol-

conjugated siRNAs can be substantially diminished or even eliminated by the presence of serum. This is likely due to binding of the cholesterol moiety with serum proteins-inhibiting the ability of the cholesterol-bound siRNAs to enter target cells. However, in the presence of a delivery enhancing agent, exemplified by the permeabilizing peptides PN73 and PN250, serum inhibition of cellular uptake can be rescued. More specifically, the addition of a permeabilizing peptide to a delivery formulation comprising a siRNA conjugated to a cholesterol moiety reduces the inhibitory effects of serum on cholesterol-siRNA uptake in a dose dependent manner.

EXAMPLE 17

Deletion Analysis of the Exemplary Polynucleotide Delivery-Enhancing Polypeptide

The present example illustrates the experimental design employed to optimize the siRNA cell-uptake and siRNA mediated target gene knockdown activities of the exemplary polynucleotide delivery-enhancing polypeptide PN73.

Table 23 below shows the exemplary polynucleotide delivery-enhancing polypeptide PN73 and truncated derivatives thereof. The amino acids sequence for PN360 and PN361 listed below are aligned with the corresponding amino acid sequence of PN73. PN360 shares its N-terminus with PN73 but lacks PN73's C-terminus while PN361 shares its C-terminus with PN73 but lacks PN73's N-terminus. PN766 represents the 15 C-terminal amino acids of PN73. PN73, PN360, PN361 and PN766 are not tagged with a C-terminal FITC (fluorescein-5-isothiocyanate) (i.e., -GK[EPSILON]G-amide). Table 23 further shows the 11 truncated forms of the exemplary polynucleotide delivery-enhancing polypeptide PN73 that were created by sequentially deleting 3 residues at a time, except PN768, from the N-terminus of the peptide. All these peptides were tagged with a C-terminus FITC (fluorescein-5-isothiocyanate) label (i.e., -GK[EPSILON]G-amide) so that cells containing the peptide could be detected by fluorescent microscopy and/or sorted by flow cytometry. Of note, PN766 and PN708 have the same amino acid sequence but differ in that PN708 has the C-terminus FITC tag. Below is an explanation of the primary structure of PN73 and the truncated forms that will be examined for transfection activity.

Table 23:

PN73 Deletion Series

C-Term. Label	Peptide ID#	SEQ ID NO:	Amino Acid Sequence
None	PN73	59	KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ-amide
	PN360	57	KGSKKAVTKAQKKDGKKRKRSRK-amide
	PN361	58	KKDGKKRKRSRKESYSVYVYKVLKQ-amide
	PN766 (PN708)	97	RKESYSVYVYKVLKQ-amide
FITC (fluorescein-5-isothiocyanate) label (i.e., -GK[EPSILON]G-amide)	PN690 (PN73)	98	KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ-GK[EPSILON-5CFG-amide
	PN661	99	KKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ-GK[EPSILON-5CFG-amide
	PN685	100	VTKAQKKDGKKRKRSRKESYSVYVYKVLKQ-GK[EPSILON-5CFG amide
	PN660	101	AQKKDGKKRKRSRKESYSVYVYKVLKQ-GK[EPSILON-5CFG-amide
	PN735	165	KDGKKRKRSRKESYSVYVYKVLKQ-GK[EPSILON-5CFG-amide
	PN655	166	KKRKRSRKESYSVYVYKVLKQ-GK[EPSILON-5CFG-amide
	PN654	167	KRSRKESYSVYVYKVLKQ-GK[EPSILON-5CFG-amide
	PN708	168	RKESYSVYVYKVLKQ-GK[EPSILON-5CFG-amide
	PN653	169	SYSVYVYKVLKQ-GK[EPSILON-5CFG-amide
	PN652	170	VYVYKVLKQ-GK[EPSILON-5CFG-amide
	PN651	171	YKVLKQ-GK[EPSILON-5CFG-amide
	PN768	172	KVLKQ-GK[EPSILON-5CFG-amide

EXAMPLE 18

Deletion Analysis of the Exemplary Polynucleotide Delivery-Enhancing Polypeptide

The functional domains of the exemplary polynucleotide delivery-enhancing polypeptide PN73 are critical to the polynucleotide delivery-enhancing polypeptide's ability to efficiently deliver siNAs into cells. These functional domains include membrane attachment, fusogenic and nucleotide binding regions. Briefly, membrane attachment describes the ability of the exemplary polynucleotide delivery-enhancing polypeptide to bind the cell membrane. The fusogenic character reflects an ability to detach from the cell membrane and enter the cytoplasm. The membrane attachment and fusogenic domains of the peptide are closely linked mechanistically (i.e., peptide's ability to enter the cell) and therefore may be difficult to differentiate

experimentally. Therefore, these two properties (domains of the peptide) will be combined into a single analysis. Lastly, the nucleotide binding describes the peptide's ability to bind nucleotides. The approaches taken to define each of the above described domains of the polynucleotide delivery-enhancing polypeptide is discussed in greater detail below. Table 24 summarizes the data for defining the membrane attachment/fusogenic and nucleotide binding domains of the exemplary polynucleotide delivery-enhancing polypeptide PN73 (the data for all concentrations tested is not shown).

Table 24:

Summary of Functional Domain Analysis of the PN73 Peptide Deletion Series

C-Term. Label	Peptide ID#	% Peptide Cell-Uptake	Peptide FITC MFI	% siRNA Cell-Uptake	siRNA Cy5 MFI	Knockdown Activity
None	PN73	N/A	N/A	**98%** (10 μM)	**13** (10 μM)	+
	PN360	N/A	N/A	**0%**	NT	NT
	PN361	N/A	N/A	**55%** (20 μM)	NT	NT
FITC (fluorescein-5-isothiocyanate) label (i.e., -GK[EPSILON]G-amide)	PN690 (PN73)	**100%** (10μM)	**125** (10 μM)	**58%** (2.5μM)	**50** (10 μM)	+
	PN661	**100%** (10 μM)	**128** (10 μM)	**49%** (2.5 μM)	**59** (10 μM)	NT
	PN685	**100%** (2.5 μM)	**151** (10 μM)	**24%** (2.5 μM)	**61** (10 μM)	NT
	PN660	**100%** (10 μM)	**121** (10 μM)	**41%** (2.5 μM)	**68** (10 μM)	+
	PN735	**100%** (10 μM)	**82** (10 μM)	**13%** (2.5 μM)	**38** (10 μM)	−
	PN655	**100%** (10 μM)	**63** (10 μM)	**14%** (10 μM)	**44** (10 μM)	NT
	PN654	**95%** (10 μM)	**10** (10 μM)	**27%** (10 μM)	**14** (10 μM)	±
	PN708	**97%** (10 μM)	**10** (10 μM)	**42%** (10 μM)	**34** (10 μM)	+
	PN653	**95%** (10 μM)	**8** (10 μM)	**1.7%** (10 μM)	**4** (10 μM)	−
	PN652	**86%** (10 μM)	**5** (10 μM)	**1.8%** (10 μM)	**5** (10 μM)	NT
	PN651	**90%** (10 μM)	**5** (10 μM)	**0%**	**3** (0.65 μM)	NT
	PN768	**91%** (50 μM)	**9** (50 μM)	NT	NT	NT

NT = not tested; peptide concentrations (in parenthesis) given are those that achieved the given uptake, in percent, or MFI in relative values.

Membrane Attachment and Fusogenic Domain of the Exemplary Polynucleotide Delivery-Enhancing Polypeptide:

The efficacy of the full-length and truncated forms of the exemplary polynucleotide delivery-enhancing polypeptide PN73 to enter cells was tested *in vitro* by a cell-uptake assay with primary mouse tail fibroblast (MTF) cells. The number of cells in culture that receive the FITC-labeled peptide was measured by flow cytometry. The percentage peptide cell-uptake was expressed relative to the total number of cells present in the culture. In addition, the Mean Fluorescence Intensity (MFI) was used to evaluate the quantity of FITC-labeled peptide found within cells. MFI directly correlates with the amount of FITC-labeled peptide within the cell: higher relative MFI value correlates with a greater amount of intracellular FITC-labeled peptides. Peptides in Table 23 were evaluated at 0.63 μM, 2.5 μM and 10 μM concentrations; PN768 was tested at 2 μM, 10 μM and 50 μM.

Full-length and truncated forms of the exemplary polynucleotide delivery-enhancing polypeptide PN73, were exposed to cells the day before transfection. FITC-tagged peptides were diluted in Opti-MEM® media (Invitrogen) for about 5 minutes at room temperature and then added to cells. Cells were transfected for 3 hours at and washed with PBS, treated with trypsin, and then analyzed by flow cytometry. Cell viability was determined as above. Cellular uptake was distinguished from the membrane insertion using trypan blue to quench any fluorescence on the cell membrane surface.

For the cell-uptake assay, the full-length FITC-labeled PN73 peptide (PN690) achieved nearly 100% cell uptake at all tested concentrations (10 μM results shown in Table 24 column entitled "% Peptide Cell-Uptake"). The remaining truncated forms of PN73, at 10 μM concentration except for PN768 which required 50 μM, achieved a percent cell uptake (values in parentheses) comparable to that of PN690 indicating that the N-terminal residues of PN73 are not required for the peptide's ability to enter cells. The five C-terminal residues of the exemplary polynucleotide delivery-enhancing polypeptide PN73, identified as PN768, are sufficient for peptide cell-uptake. However, of note and not shown in Table 24, the truncated forms of PN73 at 0.63 μM showed a decrease in cell uptake activity proportionate to the length of the peptide. In other words, the general observation of the peptides tested at a 0.63 μM concentration is that, as the PN73 peptide's length decreased, its cell uptake activity decreased thus indicating peptide cell-uptake activity is dose dependent.

These results showed that all the truncated forms of the exemplary polynucleotide delivery-enhancing polypeptide retain ability to enter a high percentage of cells in culture.

While the cell-uptake assay showed that the truncated forms of the exemplary polynucleotide delivery-enhancing polypeptide PN73 had comparable cell-uptake activities, the MFI measurements indicated that the truncated forms of the P73 peptide were distinguishable based on the mean quantity of FITC-labeled peptide that entered the cells. As shown in column entitled "Peptide FITC MIF" of Table 24, the full-length FITC-labeled PN73 peptide (PN690) showed a dose-dependent increase in MFI with 10 μM achieving the highest MFI at approximately 125 units. PN661, PN685 and PN660 had MFI levels comparable to that of the full-length PN73 (PN690). However, PN735 and PN655 had reduced MFI levels of approximately 80 MFI units and 60 MFI units, respectively. And, a significant decrease in MFI detectability was observed with PN654, PN708, PN653, PN652, PN651 and PN768 indicating that efficient uptake of the peptide by cells is ablated upon deletion of the 18 N-terminal residues of PN73.

These results showed that all truncated forms of PN73 are capable of entering a high percentage of cells in culture and, specifically, that the first 18 N-terminal residues of the exemplary polynucleotide delivery-enhancing polypeptide PN73 are essential for the efficient uptake of the peptide by cells in culture. These results showed that truncated derivative of the of the exemplary polynucleotide deliver-enhancing polypeptide PN73 (PN661; PN685; PN660; PN735; and PN655) can efficiently enter cells in culture.

In summary, the peptide cell-uptake data show that the C-terminal five residues of the exemplary polynucleotide delivery-enhancing polypeptide PN73 is sufficient for cell entry indicating that the membrane attachment/fusogenic domain of the peptide is located in the C-terminus. However, the peptide FITC MFI data show that removal of the N-terminal eighteen residues of the exemplary polynucleotide delivery-enhancing polypeptide PN73 limits the efficiency at which the peptide enters cells indicating that the N-terminus of the peptide is necessary for efficient uptake characteristic of the membrane attachment/fusogenic domain.

<u>Nucleotide Binding Domain of the Exemplary Polynucleotide Delivery-Enhancing Polypeptide:</u>

The ability of each peptide in the deletion series to complex and deliver siRNA into primary MTF cells was measured by a cell-uptake assay and by MFI. The nucleotide binding domain(s) of the exemplary polynucleotide delivery-enhancing polypeptide PN73 was characterized by comparing the relative amount of siRNA cell-uptake of the different peptides in the deletion series. Peptides either showed high percentage of siRNA cell-uptake (40% or above), which correlated with the presence of a nucleotide binding region. A low percentage of siRNA cell-uptake (below 30%) reflected the absence of the nucleotide binding domain(s).

To test the full-length and truncated forms of the exemplary polynucleotide delivery-enhancing polypeptide PN73, MTF cells were treated with Cy5- or FAM-conjugated siRNA and peptides as described above. After washing, cells were treated with trypsin, and then analyzed by flow cytometry. Intracellular uptake of siRNA was measured by the intensity of intracellular Cy5 fluorescence; trypan blue was used to quench any fluorescence on the cell membrane surface. Uptake was expressed relative to the total number of cells.

Table 24 shows the results percent siRNA cell-uptake of peptide with 0.5 μM Cy5-conjugated siRNA. The non-FITC labeled PN73 (PN643) peptide achieved nearly a 100% uptake of siRNA at 10 μM concentration (data not shown). However, when the PN73 peptide was labeled with the FITC tag (PN690), its maximum cell-uptake activity, which was observed with 2.5 μM, was reduced to approximately 60% indicating that the addition of the FITC label interferes with the peptide's cell-uptake function. Thus, the observed cell-uptake activity for each peptide in this assay may not reflect the peptide's true siRNA cell-uptake activity. Nonetheless, a slight decrease in siRNA cell-uptake activity was observed upon removal of the three most N-terminal residues of the PN73 (PN690) peptide as represented by PN661. Similarly, both PN660 and PN708 had moderate decreases in siRNA cell-uptake activity compared to the full-length PN73 (PN690).

These data show that the polynucleotide delivery-enhancing polypeptides of the invention, including PN661, PN660 and PN708, bind nucleic acids. In contrast, a reduction in siRNA uptake activity was observed with PN685, PN735, PN655 and PN654. No significant siRNA uptake activity was observed with PN653, PN652 or PN651 which suggests that the C-terminal 12 amino acids of the exemplary polynucleotide delivery-enhancing polypeptide (residues 37-48 of the H2B protein) do not contain a nucleotide binding domain. Additionally, since peptides PN661, PN660 and PN708 do not include three residue deletions of the full-length peptide (PN690) yet retain the inferred siRNA binding activity. These data suggest that the capacity of the nucleotide binding domain present within the N-terminus of the exemplary polynucleotide delivery-enhancing polypeptide to bind nucleic acids is sensitive to the presence of specific residues within the N-terminus.

In general, these data show that the siRNA cell-uptake activity of the PN73 deletion series indicates that PN708 (residues 34-48) represents the minimum C-terminal fragment of the PN73 peptide that is required for siRNA cell-uptake activity. This is consistent with the nucleotide binding domain of the exemplary polynucleotide delivery-enhancing polypeptide PN73 is located in the first 24 N-terminal residues.

The PN73 peptide deletion series were further characterized for their ability to transfect siRNAs into cells by MFI, which determined the relative mean quantity of Cy5-conjugated siRNA that entered the cells. Delivery of the Cy5-conjugated siRNA by the full-length FITC-labeled PN73 peptide (PN690) achieved a MFI of approximately 50 relative units. Peptides PN735, PN655, PN654 and PN708 of the PN73 deletion series showed a reduced MFI, ranging from approximately 34 units to 44 units. PN661, PN685 and PN660 of the PN73 deletion series had MFI levels that were slightly above that of the full-length PN73 (PN690) peptide. In contrast, PN654, PN653, PN652 and PN651 had relatively little to no MFI (3-14 units) indicating little to no Cy5-conjugated siRNA entered the cells after transfection with theses peptides. The low MFI values observed with PN653, PN652 and PN651 correlated with the siRNA cell-uptake data that showed that PN654, PN653, PN652 and PN651 do not complex with or facilitate entry of siRNA into cells.

Fluorescence microscopic imaging was used to compare the cellular localization of Cy5 labeled siRNAs complexed with the polynucleotide delivery-enhancing polypeptide PN73 to that of siRNAs transfected with lipofectamine (Invitrogen). Localization of siRNA delivered with lipofectamine is characterized by a more punctate staining, indicative of possible endosomal localization, while PN73 exhibits more uniform peri-nuclear staining. siRNA localized in endosomes is not accessible to the RISC complex in the cytoplasm, unable to silence expression of the targeted gene. In comparison, the uniform cytoplasmic distribution of siRNA observed with PN73 mediated delivery is prerequisite for access to the RISC complex, and for reducing expression of the targeted gene. The results show that the polynucleotide deliver-enhancing polypeptides of the invention substantially improve siRNA delivery over cationic lipids, and substantially improve targeted gene silencing (knock down).

These data show that the ability of the exemplary polynucleotide deliver-enhancing polypeptide PN73 to enhance the delivery of siRNA with high efficiency into cells depends on the 24 most N-terminal residues of the peptide. These data show that shorter derivatives of the exemplary polynucleotide deliver-enhancing polypeptide PN73 (PN661; PN685 PN660; PN735; PN655; and PN708) can efficiently complex with and deliver siRNA into cells.

Analysis of the Truncated Forms of Polynucleotide Delivery-Enhancing Polypeptides PN360 and PN361:

The function-structural activity relationships of the C-terminal and N-terminal regions of PN73 were shown by characterizing PN360 (C-terminal) and PN361 (N-terminal) in a siRNA cell-uptake assay performed as described above.

Table 24 shows that deleting part of the N-terminus of PN73 (see PN361) reduced siRNA cell-uptake activity by 50%; removal of C-terminal residues (see PN360) abolishes all siRNA cell-uptake activity. These data show that the C-terminal domain of the exemplary polynucleotide deliver-enhancing polypeptide PN73 is required for nucleotide cell-uptake activity of the peptide.

Peptide-siRNA conjugates Enhanced the Delivery of Covalently Linked Cargo into Cells: The peptide cell-uptake activity and MFI measurements of the truncated forms of the exemplary polynucleotide delivery-enhancing polypeptide indicate that these peptides may function as delivery vehicles for a variety of molecular cargo. This includes covalently linking the desired effector molecule, including nucleic acids and peptides to the full-length PN73 or derivates thereof. Restricted delivery of the effector molecule to a particular cell type and/or organelle within cells may be achieved by modifying the delivery peptide with a specific moiety (lipid, peptide and/or sugar group).

The deletion analysis of the exemplary polynucleotide delivery-enhancing polypeptide suggests that the N-terminus is critical to the peptide's ability to bind and deliver nucleotides (e.g., siNAs) into cells. However, even in the absence of the N-terminal residues, these non-nucleotide binding and severely compromised nucleotide binding peptides retain their membrane attachment and fusogenic domains (e.g., PN361; PN735; PN655; PN654; PN653; PN652 and PN651 and derivates thereof). In light of the peptides inability to bind nucleotides, these peptides can still be used for purposes of siNA delivery into cells by covalently linking the siNA to the peptide's membrane attachment and/or fusogenic domains. As a consequence, the siNA covalent linkage remedies the peptide's inability to bind nucleotides and allows for the efficient peptide mediated delivery of siNA into cells. Furthermore, siNA/peptide conjugates need not be limited to the select truncated forms of the exemplary polynucleotide delivery-enhancing polypeptide but may also include the full-length PN73 and derivates thereof.

The following is a non-limiting example of the methods used to generate a siNA/peptide covalent linkage. Both the peptide and siRNA molecules must be functionalized with specific moieties to allow for covalent attachment to each other. For the peptide, the N-terminus is functionalized, for example, with 3-maleimidopropionic acid. However, it is recognized that other functional groups such as bromo or iodoacetyl moieties will work as well. For the RNA molecule the 5' end of the sense strand or 3' end of the antisense strand is functionalized with, for example, a 1-O-dimethoxytrityl-hexyl-disulfide linker according to the following synthetic method.

The 5' modified siNA will be reduced to the free thiol group with 0.393 mg (3 eq) of tris(2-carboxyethyl)phosphine (TCEP) in 0.3 ml of 0.1 M triethylamine acetate (TEAA) buffer (pH 7.0) at room temperature for 3 h. The reduced oligonucleotide will be purified by RP HPLC on XTerra®MS C_{18} 4.6×50mm column using a linear gradient from 0 to 30% of CH_3CN in 0.1 M TEAA buffer pH 7 within 20 min. (t_r=5.931 min).

Such conjugates as described above were prepared. Purified reduced siNA (1.361 mg, 0.19085 μmol) was dissolved in 0.2 ml of 0.1 M TEAA buffer pH=7 and then the peptide with the maleimido moiety was attached to the peptide N-terminus (0.79 mg, 1.5 eq) and added to the siNA solution. After addition of peptide a precipitate was dissolved by addition of 150 μl of 75% CH_3CN/0.1M TEAA. After stirring overnight at room temperature, the resulting conjugate was purified by RP HPLC on XTerra®MS C_{18} 4.6×50mm column using a linear gradient from 0 to 30% of CH_3CN in 0.1M TEAA buffer pH 7 within 20 minutes and 100% C within next 5 min (t_r=21.007 min). The amount of the conjugate was determined by spectrophotometry based on the calculated molar absorption coefficient at λ=260 nm. MALDI mass spectrometric analysis showing a peak observed for the conjugate (10 585.3 amu) matching the calculated mass.

The peptide conjugate sense strand and complimentary antisense strand was annealed in 50 mM potassium acetate, 1 mM magnesium acetate and 15 mM HEPES pH 7.4 by heating at 90^0C for 2 minutes followed by incubation at 37^0C for 1 hour. The formation of the double stranded RNA conjugate was confirmed by non-denaturing (15%) polyacrylamide gel electrophoresis followed by ethidium bromide staining.

The results of cell uptake activity are shown in the following Table 25.

Table 25:

Percent of Cells that Uptake siRNA by Peptide-siRNA Conjugates

Transfection Reagent	Peptide/siRNA Conjugate Concentration	% siRNA Uptake
No Treatment	N/A	0.5%
Lipofectamine	5 ng/μl	91.9%
PN73	5 μM	92.3%
PN654 (18mer H2B)	5 μM	25.4%
	10 μM	60.3%
	20 μM	83.7%
	40 μM	87.5%
	80 μM	94.3%
	0.31 μM	6.1%
	0.63 μM	10.8%
	1.25 μM	16.5%
	2.5 μM	35.7%
	5 μM	49.8%
PN654 + Lipofectamine (5 ng/μl)	5 μM	96.2%
PN651 (6mer H2B)	0.31 μM	3.4%
	0.63 μM	11.3%
	1.25 μM	14.4%
	2.5 μM	29.6%
	5 μM	45.6%
PN651 + Lipofectamine (5 ng/μl)	5 μM	93.4%

The results in Table 25 show that the PN651-siRNA conjugate enhances uptake of siRNA into cells. Next, MFI was measured. The results are presented in Table 26.

Table 26:

Relative Quantity (MFI) of Cy5-siRNA Delivered by Peptide-siRNA Conjugates

Transfection Reagent	Peptide/siRNA Conjugate Concentration	siRNA Cy5 MFI
No Treatment	N/A	0.62
Lipofectamine	5 ng/μl	56.50
PN73	5 μM	47.70
PN654 (18mer H2B)	5 μM	3.38
	10 μM	12.20
	20 μM	28.00
	40 μM	35.50
	80 μM	68.00
	0.31 μM	1.36
	0.63 μM	1.83
	1.25 μM	2.45
	2.5 μM	4.64
	5 μM	6.87
PN654 + Lipofectamine (5 ng/μl)	5 μM	49.80
PN651 (6mer H2B)	0.31 μM	1.02
	0.63 μM	1.90
	1.25 μM	0.62
	2.5 μM	56.50
	5 μM	47.70
PN651 + Lipofectamine (5 ng/μl)	5 μM	3.38

The MFI results shown in Table 26 are consistent with the percent siRNA uptake data shown in Table 25. These data show that the PN651-siRNA conjugate enhanced siRNA uptake into cells.

<u>Gene Target Knockdown Activity of Select Truncated Forms of the Exemplary Polynucleotide Delivery-Enhancing Polypeptide:</u>

The effective knockdown of target gene expression by siRNA/polynucleotide delivery-enhancing polypeptide complexes of the invention was demonstrated. Specifically, the ability of siRNA/ polynucleotide delivery-enhancing complexes to modulate expression of the human tumor necrosis factor-α (hTNF-α) gene was assessed. The significance of targeting the hTNF-α gene is that it is implicated in mediating the occurrence or progression of rheumatoid arthritis (RA) when over-expressed in human and other mammalian subjects.

Human monocytes were used as a model system to determine the effect of siRNA/ polynucleotide delivery-enhancing complexes on hTNF-α gene expression. Qneg represents a random siRNA sequence and functioned as the negative control. The observed Qneg knockdown

activity is normalized to 100% (100% gene expression levels) and the knockdown activity of each of the following siRNAs A19S21, 21/21 and LC20 was presented as a relative percentage of the negative control. A19S21, 21/21 and LC20 are siRNAs that target hTNF-α mRNA. The exemplary polynucleotide delivery-enhancing polypeptides PN643 (full-length PN73 minus a C-terminal label), PN690 (full-length PN73 with a C-terminal FITC-label) and the truncated forms of PN73 from the deletion series, PN660, PN735, PN654 and PN708 were complexed with the A19S21, 21/21 and LC20 siRNAs to determine their effect on each siRNA's ability to reduce hTNF-α gene expression levels in human monocytes.

The experiment was performed as follows: In a 96 well flat bottom plate, human monocytes were seeded at 100K/well/100μl in OptiMEM medium (Invitrogen). Exemplary polynucleotide delivery-enhancing polypeptides were mixed with 20 nM siRNA at a molar ratio of 1 to 5 in OptiMEM medium at room temperature for 5 minutes. At the end of incubation, FBS was added to the mixture (final 3%), and 50μl of the mixture was added to the cells. The cells were incubated at 37^0C for 3 hours. After incubation, the cells were transferred to V-bottom plate and pelleted at 1500 rpm for 5 min. The cells were resuspended in growth medium (IMDM with glutamine, non-essential amino acid, and pen-strep). After an overnight incubation, the monocytes were stimulated by application of LPS (Sigma) at 1 ng/ml for 3 hours to increase expression of TNF-α expression levels. After induction by LPS, cells were collected as above for mRNA quantification, and supernatant was saved for protein quantification if desired.

For mRNA measurement, branch DNA technology from Genospectra was used according to manufacturer's specification. To quantitate mRNA level in the cells, both house keeping gene (cypB) and target gene (TNF-α) mRNA were measured, and the reading for TNF-α was normalized with cypB to obtain relative luminescence unit.

The knockdown activity for the full length and truncated forms of the exemplary polynucleotide delivery-enhancing polypeptide PN73 are summarized above in Table 24. A "+" in the "Knockdown Activity" column indicates that the peptide/siRNA complex had knockdown activity of 80% of the Qneg negative control siRNA (20% reduction in mRNA levels compared to the Qneg negative control). A "+/-" indicates that the peptide/siRNA complex had a knockdown activity of approximately 90% of the Qneg negative control siRNA (10% reduction in mRNA levels compared to the Qneg negative control). Finally, a "-" indicates that the peptide/siRNA complex had no significant knockdown activity compared to the Qneg negative control.

In general, PN643 (full-length non-FITC-labeled PN73) and PN690 (full-length FITC-labeled PN73) had equivalent siRNA knockdown activities for all siRNAs tested as indicated by "+" in the "Knockdown Activity" column (results shown in Table 24). Additionally, PN660 had siRNA knockdown activities for all siRNAs tested that were comparable to PN643 and PN690 indicating that the removal of the 9 most N-terminal residues of the PN73 peptide did not affect siRNAs mediated knockdown activity of the targeted TNF-α mRNA. PN654 showed moderate knockdown activity for both the A19S21 and 21/21 siRNAs but not for the LC20 siRNA (knockdown activity is shown by "±" in knockdown activity column). However, the siRNAs complexed with either PN708 or PN735 resulted in no observable knockdown activity for any of the siRNAs.

These results showed that truncated forms of the exemplary polynucleotide delivery-enhancing polypeptide PN73, specifically PN660 and PN654, do not interfere with siRNAs' ability to decrease mRNA levels of a target gene and are offer a new approach for improving the delivery of therapeutic siRNAs for the treatment of human diseases such as RA.

EXAMPLE 19

Characterization of the Polynucleotide Delivery-Enhancing Polypeptide PN708

The present example further explores the siRNA cell-uptake activity, MFI measurements and knockdown activity of siRNAs complexed with the PN708 peptide. Of the available peptides listed in the PN73 deletion series (refer to Table 23 in Example 17).

As described above, the cell-uptake assay determines the number of cells that receive Cy5-conjugated siRNA when complexed with a peptide. siRNA cell-uptake was assessed by flow cytometry. Uptake was expressed as a percentage calculated by dividing the number of cells containing Cy5-conjugated siRNA by the total number of transfected and untransfected cells in culture. Mean Fluorescence Intensity (MFI) was measured by flow cytometry and determined the amount of Cy5-conjugated siRNA found within cells. The MFI value directly correlates with the amount of Cy5-conjugated siRNA within the cell, thus, a higher MFI value indicates a greater number of Cy5-conjugated siRNA within the cells.

In the present example, a greater range of peptide concentrations compared to the previous example were used to determine the efficacy of siRNA cell-uptake activity and MIF measurements. Furthermore, cell viability was assessed. In the instant example, the exemplary polynucleotide delivery-enhancing polypeptides PN643 (full-length PN73 minus a C-terminal label), PN690 (full-length PN73 with a C-terminal FITC-label) and PN708 (15-mer derived by deletion of the 21 N-terminal residues of PN73) were tested at 5 μM, 10 μM, 20 μM and 40 μM.

PN643 and PN690 were also tested at 2.5 μM and PN690 was additionally tested at 1.25 μM. PN643 and PN708 were also both tested at 80 μM.

As shown in Table 27 below, the non-FITC labeled PN73 (PN643) peptide achieved nearly a 100% uptake of siRNA at 10 μM concentration. However, when the PN73 peptide was labeled with the FITC tag (PN690), its maximum cell-uptake activity was reduced to approximately 70%. PN708 showed a dose dependent increase in siRNA cell-uptake activity. PN708 achieved a maximum siRNA cell-uptake activity of 95% at 80 μM. For the full-length PN73 peptides, cell viability decreased as the concentration of peptide increased. In contrast, cells incubated with the PN708 peptide maintained over 90% cell viability in the presence of all tested concentrations. The Cy5-MFI measurements further showed that the truncated peptide PN708 practically doubled the amount of Cy5-siRNA it delivered into cells compared to the full-length PN73 (PN690) peptide.

Table 27:

Summary of siRNA Delivery-Enhancing Characteristics of PN708

Treatment	**Peptide Concentration**	**% siRNA Cell-Uptake**	**siRNA Cy5-MFI**	**% Cell Viability**
Negative Control (no treatment)	0	0	0	98%
Cy5-LC20 siRNA + PN643	2.5 μM	61%	8	95%
	5 μM	96%	13	95%
	10 μM	97%	17	94%
	20 μM	84%	10	93%
	40 μM	44%	7	78%
	80 μM	12%	14	26%
Cy5-LC20 siRNA + PN690	1.25 μM	30%	7	95%
	2.5 μM	47%	17	97%
	5 μM	71%	56	94%
	10 μM	64%	67	92%
	20 μM	55%	90	91%
	40 μM	45%	218	71%
Cy5-LC20 siRNA + PN708	5 μM	35%	9	96%
	10 μM	55%	23	96%
	20 μM	83%	85	97%
	20 μM	93%	212	94%
	80 μM	96%	378	91%

These results showed that the truncated exemplary polynucleotide delivery-enhancing polypeptide PN768 (H2B residues 34-38) has the ability to enhance the delivery of siRNA with high efficiency into cells without adversely affecting cell viability.

The truncated exemplary polynucleotide delivery-enhancing polypeptide PN708 was further characterized by determining its affect on siRNA mediated target gene expression reduction. In this section of the example, the C-terminal FITC-label of the PN708 peptide was removed prior to assessing its ability to enhance targeted gene expression reduction when complexed with a siRNA. In the absence of the FITC-label, the truncated exemplary polynucleotide delivery-enhancing polypeptide was named PN766 (refer to Table 23 in Example 17). The ability of siRNA/peptide complexes to modulate expression of the human tumor necrosis factor-α (hTNF-α) gene was assessed. In the instant example, the random siRNA sequence, Qneg, served as a negative control and the siRNAs LC20 and LC17 were used to target the hTNF-α mRNA in human monocytes. The molar ratios of siRNA to peptide tested were 1:5; 1:10; 1:25; 1:50; 1:75 and 1:100. Both LC20 and LC17 were used at 20 nM concentration.

The knockdown results show that both the LC20/PN766 and LC17/PN766 siRNA/peptide complexes at 1:5; 1:10; and 1:25 reduced hTNF-α mRNA levels to approximately 70%-80% of the Qneg siRNA negative control (i.e., 20% -30% reduction in mRNA levels compared to the Qneg negative control). The siRNA/peptide ratios of 1:50; 1:75 and 1:100 had no significant affect on hTNF-α mRNA levels compared to the Qneg control. No cytotoxicity effects were observed with human monocytes in the presence of the PN766 peptide.

These data showed that the truncated exemplary polynucleotide delivery-enhancing polypeptide PN766 when complexed with siRNA significantly reduces mRNA levels of the targeted gene indicating that PN766 is an ideal siRNA delivery peptide for therapeutic siRNAs in the treatment of RA in mammalian subjects.

EXAMPLE 20

Amino Acid Substitutions and Deletions within the Exemplary Polynucleotide Delivery Enhancing Polypeptide Do Not Affect Peptide Mediated siRNA Cell-Uptake Activity

The present example demonstrates that the mutant exemplary polynucleotide delivery-enhancing polypeptides listed in Table 28 below generated by residue substitution and/or deletion did not affect siRNA cell-uptake activity or the MFI measurements compared to the unmodified exemplary polynucleotide delivery-enhancing polypeptide PN73. Table 28 below represents the residue substitutions made within the exemplary polynucleotide delivery-enhancing polypeptide PN73. The amino acids in gray highlight represent unmodified, substituted and/or deleted residues. The gray highlight allows for easy identification and comparison of the unmodified residues within the PN73 peptide with the substituted and/or deleted residues within the mutant exemplary polynucleotide delivery-enhancing polypeptides

PN644, PN645, PN646, PN647 and PN729. Substituted residues within the mutant exemplary polynucleotide delivery-enhancing polypeptides are bolded and underlined. Furthermore, the bold and underlined symbol "^" represents a deleted residue within PN729.

The purpose of generating mutant exemplary polynucleotide delivery-enhancing polypeptides by residue substitution or deletion was to assess the effect of these modifications on siRNA cell-uptake activity and the efficiency at which siRNAs enter the cells.

Table 28:

PN73 Residue Substitution and Deletion Series

Peptide ID#	SEQ ID NO:	Amino Acid Sequence
PN73	59	KGSKKAVTKAQKKDGKKRKRSRKESYSVYVYKVLKQ
PN644	173	KGSKKAVTKAQKKDGKKRKRSRKESY**W**VYVYKVLKQ
PN645	174	KGSKKAVTKAQKKDGKKRKRSRK**W**SYSVYVYKVLKQ
PN646	175	KGSKKAVTKAQKKDGKKRKRSRK**F**SYSVYVYKVLKQ
PN647	176	KGS**F**KAVTKAQKKDGKKRKRS**F**K**F**SYSVYVYKVLKQ
PN729	177	KGS**F**KAVTKAQKK**F**GKKRKRSRK**^**S**F**SVYVYKVLKQ

X = represents a substituted amino acid; **^** = deleted amino acid;

Specific residue substitutions and/or deletions within the exemplary polynucleotide delivery-enhancing polypeptide PN73 include changing or increasing the number of aromatic amino acids and/or decreasing the number of negatively charged amino acids. Amino acids with aromatic functional groups (e.g., phenylalanine, tyrosine, tryptophan and derivatives thereof) are typically found in the membrane spanning domains of proteins due to their relatively non-polar (hydrophobic) character and ability to facilitate protein cell membrane penetration. Negatively charged amino acids repulse the negatively charged phosphodiester backbone of nucleic acids and thus impair the ability of a protein to bind nucleic acids. Thus, the rational for aromatic amino acid substitutions within the PN73 peptide include enhancing the peptide's cell penetration function and/or the removal of negatively charged amino acids to enhance the peptide's nucleic acid binding. The peptide's nucleic binding abilities may also be promoted by simply deleting negatively charged amino acids or substituting the negatively charged amino acid with a positively charged or neutral amino acid.

The siRNA cell-uptake assay and MFI measurements were performed as described previously. The data is summarized in Table 29 below. Each peptide was tested at 0.63 μM, 1.25 μM, 2.5 μM and 5 μM concentrations. The results show that despite residue substitutions and/or deletions within the exemplary polynucleotide delivery-enhancing polypeptide PN73, siRNA cell-uptake activity and MFI measurements of the mutant peptides remained equivalent to that of the unmodified PN73. These data indicate that the residue substitutions and/or deletions did not affect the peptide's ability to bind nucleic acids and penetrate the cell membrane. Furthermore, cell viability was unaffected by the presence of substituted and/or deleted residues within the exemplary polynucleotide delivery-enhancing polypeptide PN73.

Table 29:

Summary of PN73 Mutant Mediated siRNA Delivery Characteristics

Peptide ID#	Concentration	% siRNA Cell-Uptake	siRNA Cy5 MFI	% Cell Viability
No Treatment	N/A	0%	2	92%
PN73	0.63 μM	52%	2	87%
	1.25 μM	62%	4	82%
	2.5 μM	74%	14	88%
	5 μM	91%	22	93%
PN644	0.63 μM	67%	4	88%
	1.25 μM	71%	8	90%
	2.5 μM	70%	24	86%
	5 μM	83%	37	87%
PN645	0.63 μM	68%	5	84%
	1.25 μM	70%	11	89%
	2.5 μM	78%	21	90%
	5 μM	88%	28	90%
PN646	0.63 μM	67%	4	81%
	1.25 μM	70%	10	85%
	2.5 μM	73%	24	87%
	5 μM	88%	24	92%
PN647	0.63 μM	71%	13	85%
	1.25 μM	74%	34	83%
	2.5 μM	83%	39	88%

	5 µM	85%	41	87%
PN729	0.63 µM	61%	4	82%
	1.25 µM	69%	10	91%
	2.5 µM	79%	16	92%
	5 µM	86%	30	90%

These results showed that modifications of the exemplary polynucleotide delivery-enhancing polypeptide generated for example via amino acid substitutions or deletions or combinations thereof deliver siRNA to a high percentage of cells with great efficiency.

EXAMPLE 21

Polynucleotide Delivery-Enhancing Polypeptide Mediated siRNA Cell-Uptake Activity

The present example illustrates the efficacy of siRNA cell-uptake activity for the polynucleotide delivery-enhancing polypeptides listed in Table 30 complexed with siRNA. Table 31 summarizes the siRNA cell-uptake data, mean fluorescence intensity (MFI) measurements and cell viability data for each of the polypeptides. Polypeptides that achieved a percent siRNA cell-uptake of 75% or greater are highlighted in gray in the "Treatment" column. The specific percent siRNA cell-uptake for each these highlighted siRNA/peptide complexes is also highlighted in gray in the "% siRNA Cell-Uptake" column.

Table 30:

Delivery-Enhancing Polypeptides Screened for siRNA Cell-Uptake Activity

Peptide ID#	SEQ ID NO:	Amino Acid Sequence	Name
PN680	178	RSVCRQIKICRRRGGCYYKCTNRPY-amide	Androctonin
PN665	179	GFFALIPKIISSPLFKTLLSAVGSALSSSGDQE-amide	Paradaxin
PN734	180	GTAMRILGGVIPRKKRRQRRRPPQ-amide	m-Calpain + TAT
PN681	181	KKKKKRFSFKKSFKLSGFSFKKNKK-amide	MARCKS
PN694	182	RQIKIWFQNRRMKWKK-amide	Penetratin
PN714	183	RQIRIWFQNRRMRWRR-amide	PenArg
PN760	184	RKKRRQRRRPPVAYISRGGVSTYYSDTVKGRFTRQKYNKRA-amide	TAT + Peptide P3a
PN759	185	LGLLLRHLRHHSNLLANIPRKKRRQRRRPP-amide	Bindin + TAT
PN682	186	KETWWETWWTEWSQPKKKRKV-amide	Pep-1

The siRNA cell-uptake assay in the present example determines the number of cells that received Cy5-conjugated LC20 siRNA in the presence of peptide. LC20 is an oligo used for the

siRNA targeting of the human tumor necrosis factor-alpha (hTNF-α) mRNA. siRNA uptake by cells was assessed by flow cytometry. Uptake was expressed as a percentage calculated by dividing the number of cells containing Cy5-conjugated siRNA by the total number of transfected and untransfected cells in culture. Mean Fluorescence Intensity (MFI) was measured by flow cytometry and determined the amount of Cy5-conjugated siRNA found within cells. The MFI value directly correlates with the amount of Cy5-conjugated siRNA within the cell, thus, a higher MFI value indicates a greater number of Cy5-conjugated siRNA within the cells.

The following protocol was used to test the polynucleotide delivery-enhancing polypeptides listed in Table 30. Approximately 80,000 mouse tail fibroblast (MTF) cells were plated per well in 24-well plates the day before transfection in complete media. Each delivery peptide, except the positive control, was tested at 0.63 μM, 2.5 μM, 10 μM and 40 μM concentrations in the presence of 0.5 μM Cy5-conjugated siRNA. For siRNA/peptide complexes, the Cy5-conjugated siRNA and peptide were diluted separately in Opti-MEM® media (Invitrogen) at two-fold the final concentration. Equal volumes of siRNA and peptide were mixed and allowed to complex five minutes at room temperature. The siRNA/peptide complexes were added to cells previously washed with phosphate buffered saline (PBS). Cells were transfected for three hours at 37°C, 5% CO_2. Cells were washed with PBS, treated with trypsin, and then analyzed by flow cytometry. siRNA cell-uptake was measured by the intensity of intracellular Cy5 fluorescence. Cell viability was determined using propidium iodide uptake or AnnexinV-PE (BD Biosciences) staining. In order to differentiate the cellular uptake from the membrane insertion of labeled siRNA (or fluorescein-labeled peptide), trypan blue was used to quench any fluorescence on the cell membrane surface. Trypan blue (Sigma) was added to cells to a final concentration of 0.04% and re-run on the flow cytometer to assess whether there was any change in fluorescence intensity which would indicate fluorescence localized to the cell membrane.

Table 31:

Data of Polypeptide Mediated siRNA Delivery Screen (NT = not tested)

Treatment (siRNA/Polypeptide Complex)	Peptide Concentration	% siRNA Cell-Uptake	Cy5-siRNA MFI	% Cell Viability
No treatment	N/A	0.0%	0.0	97.6%
Cy5-LC20 + PN643 (positive control)	5 μM	95.4%	7.2	98.8%
Cy5-LC20 + PN680	0.63 μM	0.2%	N/T	98.2%
	2.5 μM	1.8%	1.4	98.3%
	10 μM	82.6%	4.5	99.2%
	40 μM	79.1%	5.2	95.7%
Cy5-LC20 + PN665	0.63 μM	0.0%	N/T	97.7%
	2.5 μM	0.6%	N/T	95.1%
	10 μM	N/T	N/T	N/T
	40 μM	N/T	N/T	N/T
Cy5-LC20 + PN734	0.63 μM	0.1%	N/T	98.2%
	2.5 μM	0.2%	N/T	98.7%
	10 μM	1.2%	1.3	98.4%
	40 μM	4.5%	1.6	97.0%
Cy5-LC20 + PN681	0.63 μM	0.2%	1.8	97.1%
	2.5 μM	69.9%	4.6	98.9%
	10 μM	97.3%	15.3	98.2%
	40 μM	91.2%	13.7	92.6%
Cy5-LC20 + PN694	0.63 μM	0.2%	1.4	97.1%
	2.5 μM	0.2%	1.8	97.9%
	10 μM	48.0%	4.2	97.8%
	40 μM	54.0%	3.9	83.6%
Cy5-LC20 + PN714	0.63 μM	0.4%	1.2	95.1%
	2.5 μM	0.5%	2.3	96.4%
	10 μM	19.1%	2.5	97.6%
	40 μM	43.0%	4.9	94.7%
Cy5-LC20 + PN709	0.63 μM	0.1%	1.0	94.0%
	2.5 μM	0.2%	1.0	96.6%
	10 μM	18.6%	1.9	97.1%
	40 μM	76.6%	5.8	97.1%
Cy5-LC20 + PN760	0.63 μM	60%	2.9	84.7%
	2.5 μM	85.5%	78.5	90.8%
	10 μM	90.6%	96.9	91.9%
	40 μM	82.8%	77.4	83.2%
Cy5-LC20 + PN759	0.63 μM	43%	2.1	81.7%
	2.5 μM	72.9%	7.3	85.2%
	10 μM	83.6%	40.9	86.7%
	40 μM	25%	10.5	26.6%
Cy5-LC20 + PN682	0.63 μM	52.1%	2.4	86.2%
	2.5 μM	50.6%	2.2	91.3%
	10 μM	56.9%	2.3	90.6%
	40 μM	92%	9.0	97.1%

As shown in the column entitled "% siRNA Cell-Uptake" of Table 31, the "no treatment" negative control showed no siRNA cell-uptake while the positive control peptide achieved a percent siRNA cell-uptake activity of 95%. The Cy5 conjugated LC20 siRNA complexed with the polynucleotide delivery-enhancing polypeptides PN680; PN681; PN709; PN760; PN759 or PN682 achieved a percent siRNA cell-uptake activity that exceeded 75% or greater. The polynucleotide delivery-enhancing polypeptides PN694 and PN714 exhibited a moderate siRNA cell-uptake activity of 54% and 43%, respectively. In contrast, the polynucleotide delivery-enhancing polypeptides PN665 and PN734 demonstrated no significant siRNA cell-uptake activity (less than 5%).

The polynucleotide delivery-enhancing polypeptides were further characterized for their ability to transfect siRNAs into cells by analyzing Mean Fluorescence Intensity (MFI). While the cell-uptake assay determined the percentage of cells that contain the Cy5-conjugated siRNA, the MFI measurement determined the relative mean quantity of Cy5-conjugated siRNA that entered the cells. As shown in the column entitled "siRNA Cy5 MFI" of Table 31, delivery of the Cy5-conjugated siRNA by the positive control peptide PN643 achieved a MFI of approximately seven units. As expected, the "no treatment" negative control has no measurable MFI. The polynucleotide delivery-enhancing polypeptide PN665 was not tested by MFI. PN743, PN694 and PN714 had MFI measurements significantly lower than that of the positive control. The polynucleotide delivery-enhancing polypeptides PN680, PN709 and PN682 exhibited MFI measurements comparable to that of the PN643 positive control while PN681 had an MFI double that of the positive control. Surprisingly, the polynucleotide delivery-enhancing polypeptides PN760 and PN759 had MFI measurements that were approximately 13-fold and 6-fold greater, respectively, than that of the positive control.

These data show that the polynucleotide deliver-enhancing polypeptides PN680; PN681; PN709; PN760; PN759 and PN682 when complexed with siRNA efficiently deliver siRNA into cells.

EXAMPLE 22

Gene Expression Knockdown Activity of siRNAs Transfected Into Cells with Polynucleotide Delivery-Enhancing Polypeptides

The present example demonstrates that siRNA complexed with polynucleotide delivery-enhancing polypeptides effectively knockdown mRNA expression of the siRNA targeted gene. Specifically, the ability of siRNA/peptide complexes to modulate expression of the human tumor necrosis factor-α (hTNF-α) gene was assessed. The significance of targeting the hTNF-α gene

is that it is implicated in mediating the occurrence or progression of rheumatoid arthritis (RA) when over-expressed in human and other mammalian subjects.

Human monocytes were used as a model system to determine the effect of siRNA/peptide complexes on hTNF-α gene expression. Qneg represents a random siRNA sequence and functioned as the negative control. The observed Qneg knockdown activity is normalized to 100% (100% gene expression levels) and the knockdown activity for each of the following siRNAs A19S21 MD8, 21/21 MD8 and LC20 was presented as a relative percentage of the negative control. A19S21 MD8, 21/21 MD8 and LC20 are siRNAs that target hTNF-α mRNA.

The polynucleotide delivery-enhancing polypeptide PN602 represented an acetylated form of the positive control used in prior Examples and is used herein as a positive control for both the effective delivery of siRNA into human monocytes and the permissive knockdown activity of hTNF-α mRNA levels mediated by siRNA. The polynucleotide delivery-enhancing polypeptides PN680 and PN681 were complexed with the above listed siRNAs to determine their effect on each siRNA's ability to reduce hTNF-α gene expression levels in human monocytes. The knockdown activity of all three polynucleotide delivery-enhancing polypeptides is summarized below in Table 32. A "+" in the "Knockdown Activity" column indicates that the peptide/siRNA complex had knockdown activity of 80% of the Qneg negative control siRNA (20% reduction in mRNA levels compared to the Qneg negative control). A "+/-" indicates that the peptide/siRNA complex had a knockdown activity of approximately 90% of the Qneg negative control siRNA (10% reduction in mRNA levels compared to the Qneg negative control). Finally, a "-" indicates that the peptide/siRNA complex had no significant knockdown activity compared to the Qneg negative control.

Transfections in human monocytes for the instant example was performed according to protocols previously described.

For mRNA measurement, branch DNA technology from Genospectra (CA) was used according to manufacturer's specification. To quantitate mRNA level in the cells, both house keeping gene (cypB) and target gene (TNF-α) mRNA were measured, and the reading for TNF-α was normalized with cypB to obtain relative luminescence unit.

Table 32:
siRNA Knockdown Activity for siRNAs
Complexed with Polynucleotide Delivery-Enhancing Polypeptides

Peptide ID #	**siRNA:Peptide Ratio**	**siRNA**		
		A19S21 MD8	21/21 MD8	LC20
PN602 (Positive control)	1:5	+/-	+/-	+/-
	1:10	+/-	+/-	+/-
PN680	1:5	+	+	+
	1:10	+/-	+/-	+
PN681	1:5	+/-	-	-
	1:10	+/-	-	-

The results shown in Table 32 indicate that all three siRNAs complexed with the positive control PN602 polynucleotide delivery-enhancing polypeptide at ratios of 1:5 and 1:10 moderately reduced hTNF-α gene expression levels compared to the Qneg negative control complexed with the same polypeptide. However, the same siRNAs complexed with the polynucleotide delivery-enhancing polypeptide PN681 at 1:5 and 1:10 showed little to no knockdown activity relative to the Qneg negative control siRNA/PN681 complex. In contrast, the polynucleotide delivery-enhancing polypeptide PN680 complexed with any of the hTNF-α specific siRNAs at a 1:5 ratio exhibited significant knockdown activity of the hTNF-α mRNA relative to the Qneg/PN680 control complex. Furthermore, the LC20/PN680 complex at a 1:10 ratio also demonstrated significant knockdown activity compared to the Qneg/PN680 control complex.

These data show that the polynucleotide delivery-enhancing polypeptide PN680 delivers siRNAs into cells and permits effective siRNA mediated gene silencing.

Although the foregoing invention has been described in detail by way of example for purposes of clarity of understanding, it will be apparent to the artisan that certain changes and modifications may be practiced within the scope of the appended claims which are presented by way of illustration not limitation. In this context, various publications and other references have been cited within the foregoing disclosure for economy of description. Each of these references is incorporated herein by reference in its entirety for all purposes. It is noted, however, that the various publications discussed herein are incorporated solely for their disclosure prior to the filing date of the present application, and the inventors reserve the right to antedate such disclosure by virtue of prior invention.

WHAT IS CLAIMED IS:

1. A composition comprising a polynucleotide delivery-enhancing polypeptide and a double stranded ribonucleic acid (dsRNA), wherein the polynucleotide delivery-enhancing polypeptide is amphipathic and comprises nucleic acid binding properties.

2. The composition of Claim 1, wherein the polynucleotide delivery-enhancing polypeptide comprises about 5 to about 40 amino acids, and has all or part of a sequence selected from the group consisting of Poly (Lys, Tryp) 4:1 MW 20,000-50,000, Poly (Orn, Trp) 4:1 20,000-50,000, Mellitin, Histone H1, Histone H3 and Histone H4, SEQ ID NOS 27 to 31, 35 to 42, 45, 47, 50 to 59, 62, 63, 67, 68, 73, 74, 76, 78 to 87, 89 to 92, 94 to 108, 164 to 178 and 180 to 186.

3. The composition of Claim 1, wherein the composition causes uptake of the dsRNA into an animal cell.

4. The composition of the Claim 3, wherein the animal cell is a mammalian cell.

5. The composition of Claim 1, wherein the composition is administered to an animal.

6. The composition of the Claim 5, wherein the animal is a mammal.

7. The composition of Claim 1, wherein the N-terminus of the polynucleotide delivery-enhancing polypeptide is acetylated.

8. The composition of Claim 1, wherein the N-terminus of the polynucleotide delivery-enhancing polypeptide is pegylated.

9. The composition of Claim 1, wherein the dsRNA is a small interfering ribonucleic acid (siRNA) consisting of about 10 to about 40 base pair sequence that is complementary to a portion of a Tumor Necrosis Factor-alpha (TNF-α) gene.

10. The composition of Claim 1, wherein the dsRNA is a siRNA consisting of about 10 to about 40 base pair sequence selected from the group consisting of SEQ ID NOS 109 to 163 and 187.

11. The composition of Claim 1, wherein the polynucleotide delivery-enhancing polypeptide is admixed, complexed or conjugated to the dsRNA.

12. The composition of Claim 1, wherein the polynucleotide delivery-enhancing polypeptide binds to the dsRNA.

13. The composition of Claim 1, further comprising a cationic lipid.

14. The composition of Claim 13, wherein the cationic lipid is selected from the group consisting of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride, 1,2-bis(oleoyloxy)-3-3-(trimethylammonium)propane, 1,2-dimyristyloxypropyl-3-dimethylhydroxyethylammonium bromide, dimethyldioctadecylammonium bromide, 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminiu m trifluoracetate, 1,3-dioleoyloxy-2-(6-carboxyspermyl)-propylamid, 5-carboxyspermylglycine dioctadecylamide, tetramethyltetrapalmitoyl spermine, tetramethyltetraoleyl spermine, tetramethyltetralauryl spermine, tetramethyltetramyristyl spermine and tetramethyldioleyl spermine, DOTMA (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride), DOTAP (1,2-bis(oleoyloxy)-3,3-(trimethylammonium)propane), DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide), DDAB (dimethyl dioctadecyl ammonium bromide), polyvalent cationic lipids, lipospermines, DOSPA (2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanamini um trifluoro-acetate), DOSPER (1,3-dioleoyloxy-2-(6carboxy spermyl)-propyl-amid, di- and tetra-alkyl-tetra-methyl spermines, TMTPS (tetramethyltetrapalmitoyl spermine), TMTOS (tetramethyltetraoleyl spermine), TMTLS (tetramethlytetralauryl spermine), TMTMS (tetramethyltetramyristyl spermine), TMDOS (tetramethyldioleyl spermine) DOGS (dioctadecyl-amidoglycylspermine (TRANSFECTAM®), cationic lipids combined with non-cationic lipids, DOPE (dioleoylphosphatidylethanolamine), DPhPE (diphytanoylphosphatidylethanolamine) or cholesterol, a cationic lipid composition composed of a 3:1 (w/w) mixture of DOSPA and DOPE, and a 1:1 (w/w) mixture of DOTMA and DOPE.

15. A method for causing uptake of a double stranded ribonucleic acid (dsRNA) into an animal cell, which comprises incubating the animal cells with a mixture comprising a polynucleotide delivery-enhancing polypeptide and the dsRNA, wherein the polynucleotide delivery-enhancing polypeptide is amphipathic and comprises nucleic acid binding properties.

16. A method for modifying expression of a target gene in an animal cell, which comprises incubating the animal cell with a mixture comprising a polynucleotide delivery-

enhancing polypeptide, wherein the polynucleotide delivery-enhancing polypeptide is amphipathic and comprises nucleic acid binding properties, and a double stranded ribonucleic acid (dsRNA), wherein the dsRNA is complementary to a region of the target gene.

17. The method of Claim 15 or 16, wherein the animal cell is a mammalian cell.

18. A method for changing a phenotype of an animal subject, which comprises administering to the animal subject a mixture of a polynucleotide delivery-enhancing polypeptide, wherein the polynucleotide delivery-enhancing polypeptide is amphipathic and comprises nucleic acid binding properties, and a double stranded ribonucleic acid (dsRNA), wherein the dsRNA is complementary to a region of a target gene in the subject.

19. The method of Claim 18, wherein the animal is a mammal.

20. The method of Claim 15, 16 or 18, wherein the polynucleotide delivery-enhancing polypeptide comprises about 5 to about 40 amino acids, and has all or part of a sequence selected from the group consisting of Poly (Lys, Tryp) 4:1 MW 20,000-50,000, Poly (Orn, Trp) 4:1 20,000-50,000, Mellitin, Histone H1, Histone H3 and Histone H4, SEQ ID NOS 27 to 31, 35 to 42, 45, 47, 50 to 59, 62, 63, 67, 68, 73, 74, 76, 78 to 87, 89 to 92, 94 to 108, 164 to 178 and 180 to 186.

21. The method of Claim 15, 16 or 18, wherein the N-terminus of the polynucleotide delivery-enhancing polypeptide is acetylated.

22. The method of Claim 15, 16 or 18, wherein the N-terminus of the polynucleotide delivery-enhancing polypeptide is pegylated.

23. The method of Claim 15, 16 or 18, wherein the dsRNA is a small interfering ribonucleic acid (siRNA) consisting of about 10 to about 40 base pair sequence that is complementary to a portion of a Tumor Necrosis Factor-alpha (TNF-α) gene.

24. The method of Claim 15, 16 or 18, wherein the dsRNA is a siRNA consisting of about 10 to about 40 base pair sequence selected from the group consisting of SEQ ID NOS 109 to 163 and 187.

25. The method of Claim 15, 16 or 18, wherein the polynucleotide delivery-enhancing polypeptide is admixed, complexed or conjugated to the dsRNA.

26. The method of Claim 15, 16 or 18, wherein the polynucleotide delivery-enhancing polypeptide binds to the dsRNA.

27. The method of Claim 15, 16 or 18, further comprising a cationic lipid.

28. The method of Claim 27, wherein the cationic lipid is selected from the group consisting of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride, 1,2-bis(oleoyloxy)-3-3-(trimethylammonium)propane, 1,2-dimyristyloxypropyl-3-dimethylhydroxyethylammonium bromide, dimethyldioctadecylammonium bromide, 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminiu m trifluoracetate, 1,3-dioleoyloxy-2-(6-carboxyspermyl)-propylamid, 5-carboxyspermylglycine dioctadecylamide, tetramethyltetrapalmitoyl spermine, tetramethyltetraoleyl spermine, tetramethyltetralauryl spermine, tetramethyltetramyristyl spermine and tetramethyldioleyl spermine, DOTMA (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl ammonium chloride), DOTAP (1,2-bis(oleoyloxy)-3,3-(trimethylammonium)propane), DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide), DDAB (dimethyl dioctadecyl ammonium bromide), polyvalent cationic lipids, lipospermines, DOSPA (2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanamini um trifluoro-acetate), DOSPER (1,3-dioleoyloxy-2-(6carboxy spermyl)-propyl-amid, di- and tetra-alkyl-tetra-methyl spermines, TMTPS (tetramethyltetrapalmitoyl spermine), TMTOS (tetramethyltetraoleyl spermine), TMTLS (tetramethlytetralauryl spermine), TMTMS (tetramethyltetramyristyl spermine), TMDOS (tetramethyldioleyl spermine) DOGS (dioctadecyl-amidoglycylspermine (TRANSFECTAM®), cationic lipids combined with non-cationic lipids, DOPE (dioleoylphosphatidylethanolamine), DPhPE (diphytanoylphosphatidylethanolamine) or cholesterol, a cationic lipid composition composed of a 3:1 (w/w) mixture of DOSPA and DOPE, and a 1:1 (w/w) mixture of DOTMA and DOPE.

29. A use of a mixture comprising a polynucleotide delivery-enhancing polypeptide, wherein the polynucleotide delivery-enhancing polypeptide is amphipathic and comprises nucleic acid binding properties, and a double stranded ribonucleic acid (dsRNA) for the production of a medicament for the treatment of a Tumor Necrosis Factor-alpha (TNF-α) associated inflammatory condition(s) in an animal subject, wherein the medicament is capable of reducing TNF-α RNA levels thereby preventing or reducing the occurrence or severity of one or more symptom(s) of the TNF-α associated inflammatory condition(s).

30. The use of a mixture of Claim 29, wherein the polynucleotide delivery-enhancing polypeptide comprises about 5 to about 40 amino acids, and has all or part of a sequence selected from the group consisting of Poly (Lys, Tryp) 4:1 MW 20,000-50,000, Poly (Orn, Trp) 4:1 20,000-50,000, Mellitin, Histone H1, Histone H3 and Histone H4, SEQ ID NOS 27 to 31, 35 to 42, 45, 47, 50 to 59, 62, 63, 67, 68, 73, 74, 76, 78 to 87, 89 to 92, 94 to 108, 164 to 178 and 180 to 186.

31. The use of a mixture of Claim 29, wherein the N-terminus of the polynucleotide delivery-enhancing polypeptide is acetylated.

32. The use of a mixture of Claim 29, wherein the N-terminus of the polynucleotide delivery-enhancing polypeptide is pegylated.

33. The use of a mixture of Claim 29, wherein the dsRNA is a small interfering ribonucleic acid (siRNA) consisting of about 10 to about 40 base pair sequence that is complementary to a portion of a Tumor Necrosis Factor-alpha (TNF-α) gene.

34. The use of a mixture of Claim 29, wherein the dsRNA is a siRNA consisting of about 10 to about 40 base pair sequence selected from the group consisting of SEQ ID NOS 109 to 163 and 187.

35. The use of a mixture of Claim 29, wherein the polynucleotide delivery-enhancing polypeptide is admixed, complexed or conjugated to the dsRNA.

36. The use of a mixture of Claim 29, wherein the polynucleotide delivery-enhancing polypeptide binds to the dsRNA.

37. The use of a mixture of Claim 29, wherein the animal subject is a mammal.

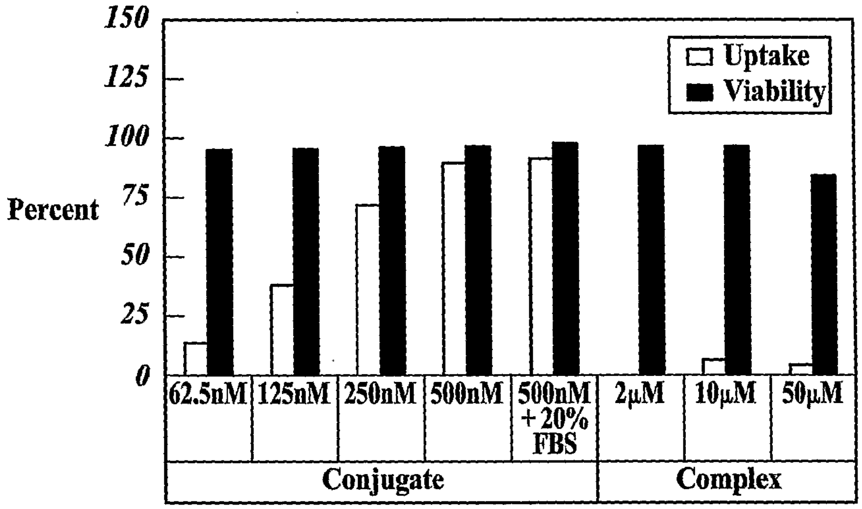

150
125
100
75
50
25
0
Percent
Uptake
Viability
62.5nM
125nM
250nM
500nM
500nM + 20% FBS
2μM
10μM
50μM
Conjugate
Complex

FIG.1

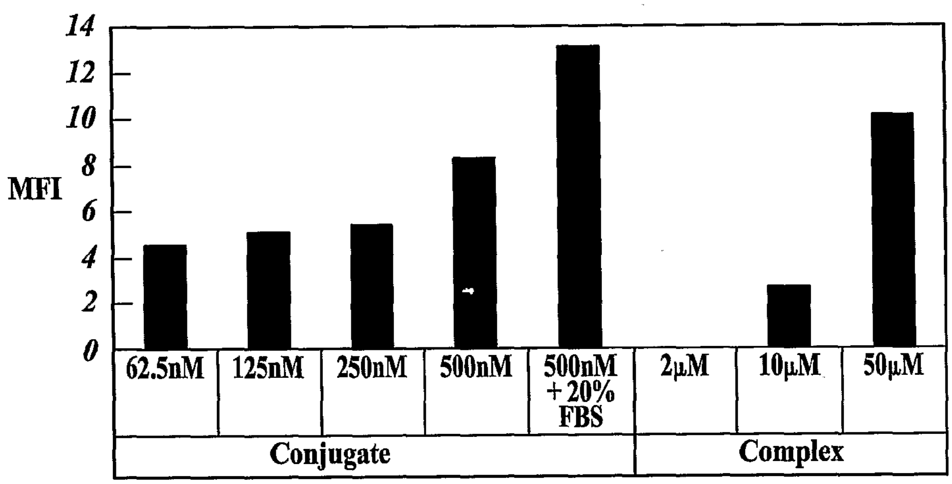
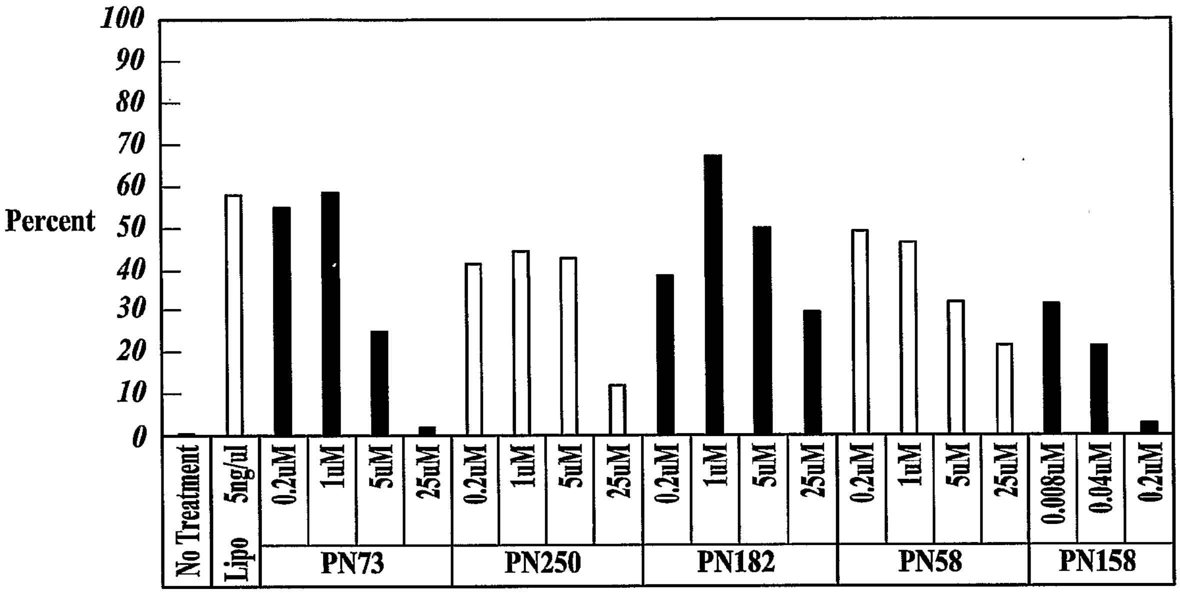

14
12
10
8
6
4
2
0
MFI
62.5nM
125nM
250nM
500nM
500nM +20% FBS
2μM
10μM
50μM
Conjugate
Complex

FIG.2



100
90
80
70
60
Percent
50
40
30
20
10
0
No Treatment
Lipo
5ng/ul
0.2uM
1uM
5uM
25uM
PN73
0.2uM
1uM
5uM
25uM
PN250
0.2uM
1uM
5uM
25uM
PN182
0.2uM
1uM
5uM
25uM
PN58
0.008uM
0.04uM
0.2uM
PN158

FIG.3

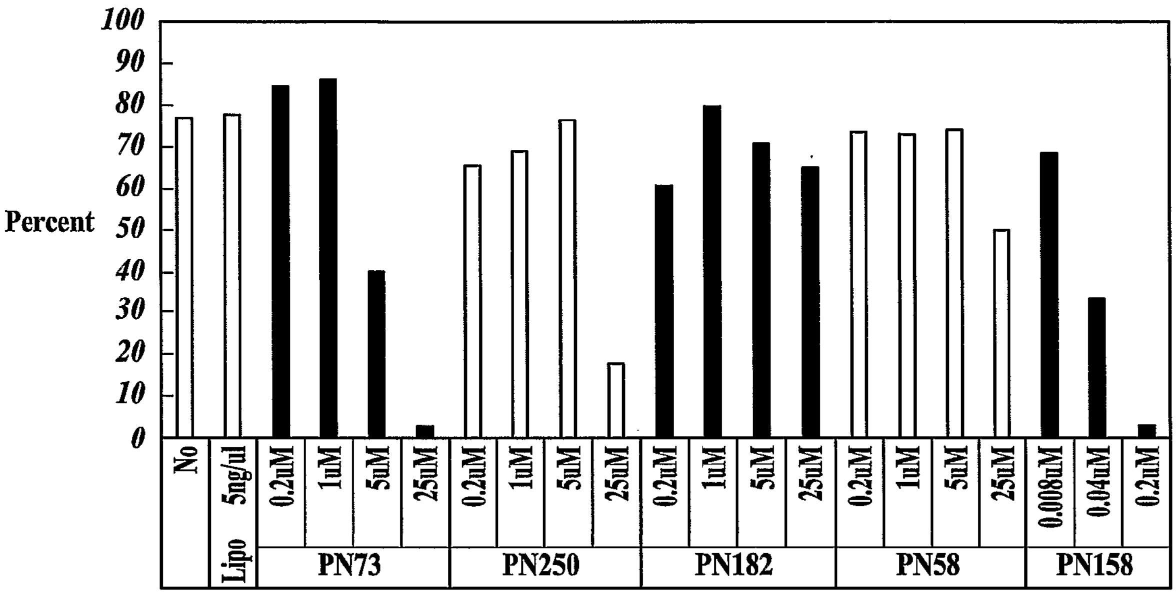

100
90
80
70
60
Percent
50
40
30
20
10
0
No
5ng/ul
Lipo
0.2uM
1uM
5uM
25uM
PN73
PN250
PN182
PN58
0.008uM
0.04uM
0.2uM
PN158

FIG.4

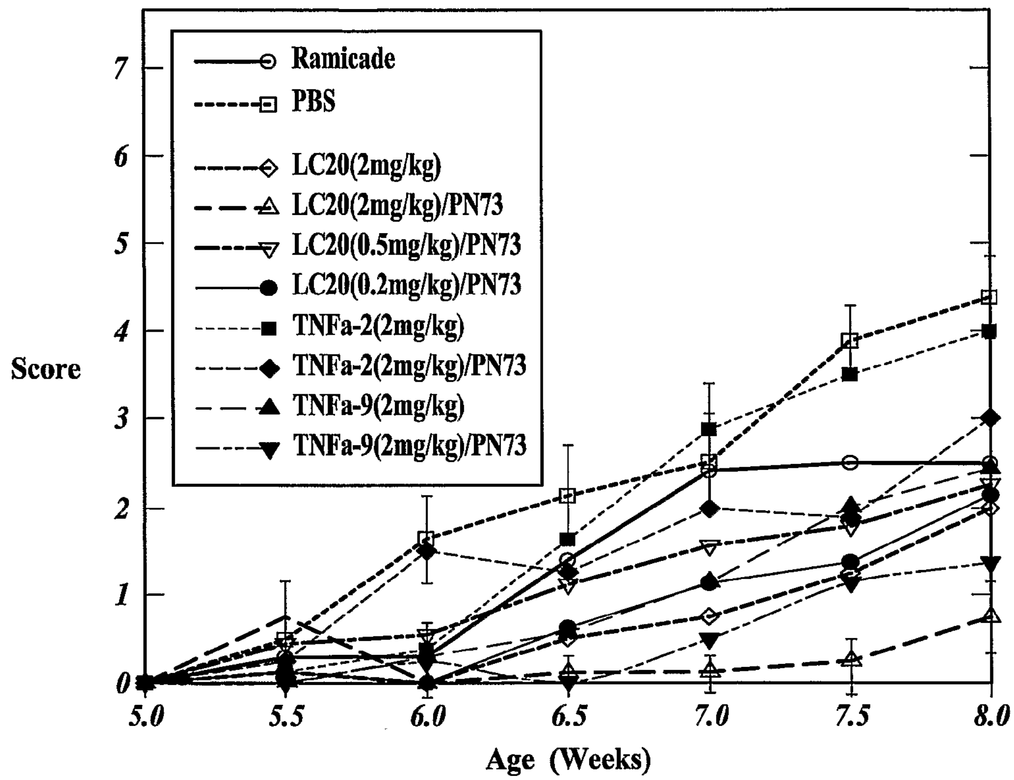

Ramicade
PBS
LC20(2mg/kg)
LC20(2mg/kg)/PN73
LC20(0.5mg/kg)/PN73
LC20(0.2mg/kg)/PN73
TNFa-2(2mg/kg)
TNFa-2(2mg/kg)/PN73
TNFa-9(2mg/kg)
TNFa-9(2mg/kg)/PN73
Score
0
1
2
3
4
5
6
7
5.0
5.5
6.0
6.5
7.0
7.5
8.0
Age (Weeks)

FIG. 5

6/10

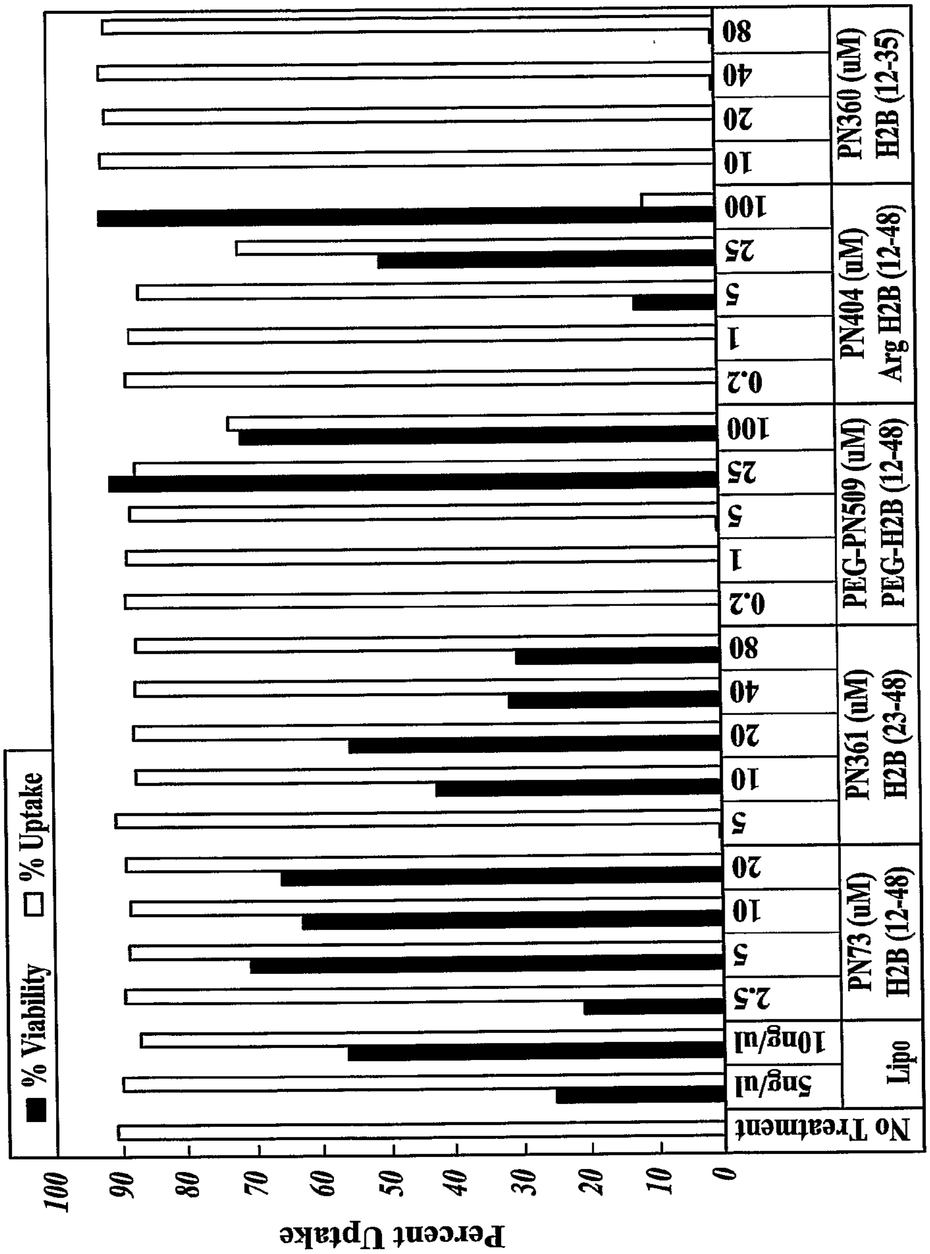

■ % Viability
□ % Uptake
Percent Uptake
0
10
20
30
40
50
60
70
80
90
100
No Treatment
5ng/ul
10ng/ul
Lipo
2.5
5
10
20
PN73 (uM)
H2B (12-48)
5
10
20
40
80
PN361 (uM)
H2B (23-48)
0.2
1
5
25
100
PEG-PN509 (uM)
PEG-H2B (12-48)
0.2
1
5
25
100
PN404 (uM)
Arg H2B (12-48)
10
20
40
80
PN360 (uM)
H2B (12-35)

FIG.6

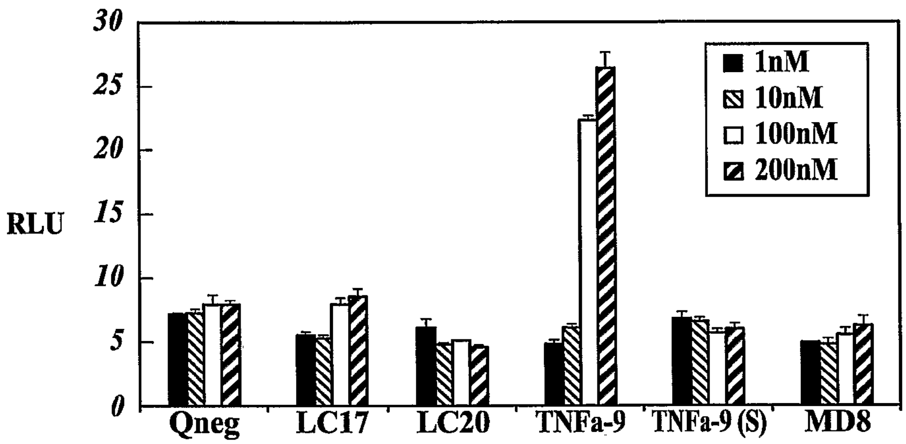

30
25
20
15
10
5
0
RLU
1nM
10nM
100nM
200nM
Qneg
LC17
LC20
TNFa-9
TNFa-9 (S)
MD8

FIG. 7A

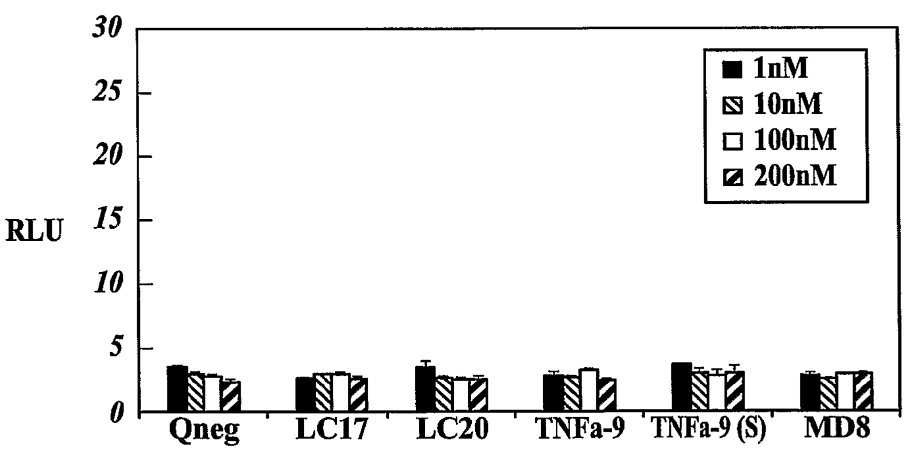

30
25
20
15
10
5
0
RLU
1nM
10nM
100nM
200nM
Qneg
LC17
LC20
TNFa-9
TNFa-9 (S)
MD8

FIG. 7B

FIG. 8A

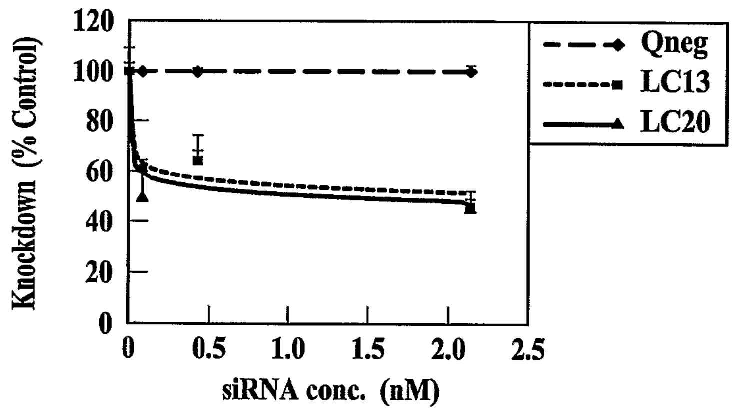


FIG. 8B

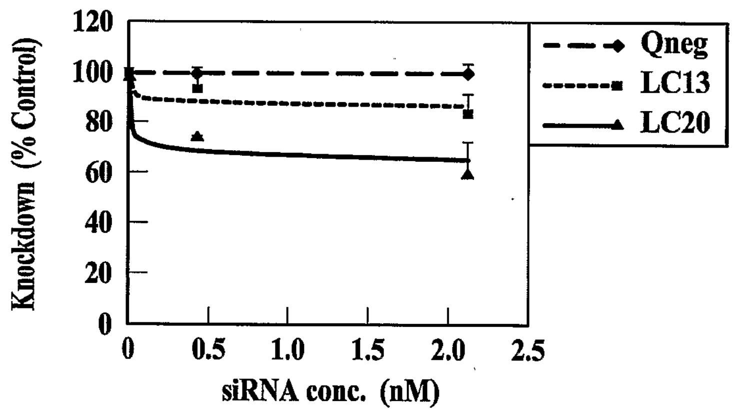
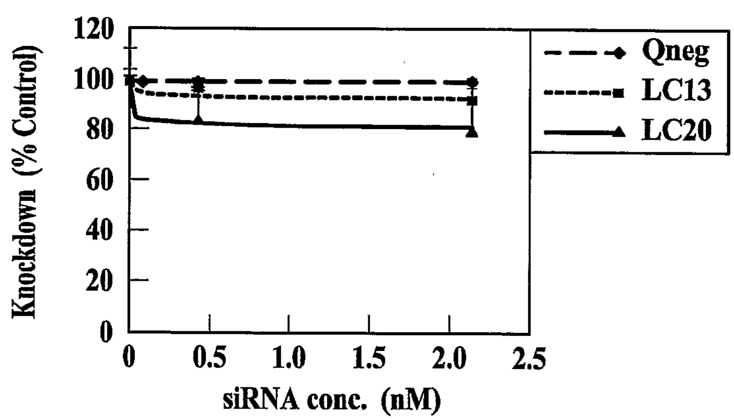
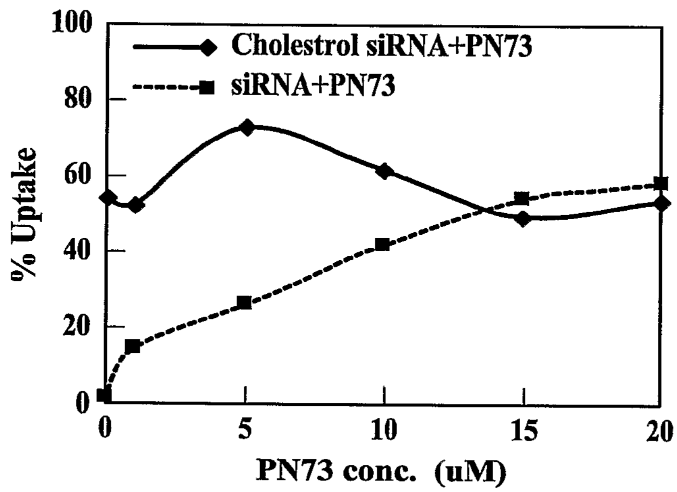


FIG. 8C





100
80
60
40
20
0
% Uptake
Cholestrol siRNA+PN73
siRNA+PN73
0
5
10
15
20
PN73 conc. (uM)

FIG.9

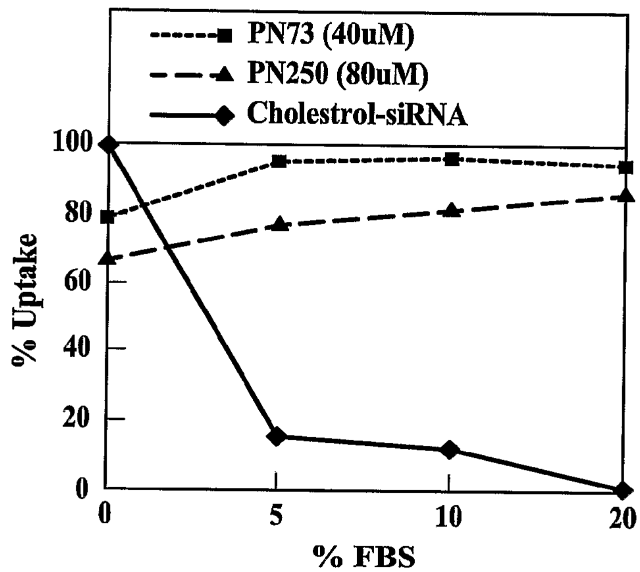

PN73 (40uM)
PN250 (80uM)
Cholestrol-siRNA
% Uptake
100
80
60
40
20
0
0
5
10
20
% FBS

FIG.10

SEQUENCE LISTING

<110> NASTECH PHARMACEUTICAL COMPANY, INC.

<120> PHARMACEUTICAL COMPOSITIONS FOR DELIVERY OF RIBONUCLEIC ACID TO A CELL

<130> 04-03CIP-PCT

<140>
<141>

<150> 11/223,699
<151> 2005-09-08

<150> 60/727,216
<151> 2005-10-14

<150> 60/733,664
<151> 2005-11-04

<160> 188

<170> PatentIn Ver. 3.3

<210> 1
<211> 7
<212> PRT
<213> Human immunodeficiency virus

<400> 1
Lys Arg Arg Gln Arg Arg Arg
1 5

<210> 2
<211> 16
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Unknown Penetratin PTD peptide

<400> 2
Arg Gln Ile Lys Ile Trp Phe Gln Asn Arg Arg Met Lys Trp Lys Lys
1 5 10 15

<210> 3
<211> 34
<212> PRT
<213> herpes simplex virus

<400> 3
Asp Ala Ala Thr Ala Thr Arg Gly Arg Ser Ala Ala Ser Arg Pro Thr
1 5 10 15

Glu Arg Pro Arg Ala Pro Ala Arg Ser Ala Ser Arg Pro Arg Arg Pro
20 25 30

Val Asp

<210> 4
<211> 16
<212> PRT
<213> Homo sapiens

<400> 4
Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro
1 5 10 15

<210> 5
<211> 16
<212> PRT
<213> Homo sapiens

<400> 5
Ala Ala Val Leu Leu Pro Val Leu Leu Pro Val Leu Leu Ala Ala Pro
1 5 10 15

<210> 6
<211> 15
<212> PRT
<213> Homo sapiens

<400> 6
Val Thr Val Leu Ala Leu Gly Ala Leu Ala Gly Val Gly Val Gly
1 5 10 15

<210> 7
<211> 17
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: gp41 fusion peptide

<400> 7
Gly Ala Leu Phe Leu Gly Trp Leu Gly Ala Ala Gly Ser Thr Met Gly
1 5 10 15

Ala

<210> 8
<211> 17
<212> PRT
<213> Caiman crocodilus

<400> 8
Met Gly Leu Gly Leu His Leu Leu Val Leu Ala Ala Ala Leu Gln Gly
1 5 10 15

Ala

<210> 9
<211> 24
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: hCT-derived
peptide

<400> 9
Leu Gly Thr Tyr Thr Gln Asp Phe Asn Lys Phe His Thr Phe Pro Gln
1 5 10 15

Thr Ala Ile Gly Val Gly Ala Pro
20

<210> 10
<211> 26
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Unknown
transportan peptide

<400> 10
Gly Trp Thr Leu Asn Ser Ala Gly Tyr Leu Leu Lys Ile Asn Leu Lys
1 5 10 15

Ala Leu Ala Ala Leu Ala Lys Lys Ile Leu
20 25

<210> 11
<211> 16
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Unknown loligomer
peptide

<400> 11
Thr Pro Pro Lys Lys Lys Arg Lys Val Glu Asp Pro Lys Lys Lys Lys
1 5 10 15

<210> 12
<211> 7
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Unknown Arginine
peptide

<400> 12
Arg Arg Arg Arg Arg Arg Arg
1 5

<210> 13
<211> 18
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Amphiphilic
model peptide

<400> 13
Lys Leu Ala Leu Lys Leu Ala Leu Lys Ala Leu Lys Ala Ala Leu Lys
1 5 10 15

Leu Ala

<210> 14
<211> 16
<212> PRT
<213> Influenza virus

<400> 14
Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile Glu Asn Gly Trp Glu Gly
1 5 10 15

<210> 15
<211> 16
<212> PRT
<213> Sendai virus

<400> 15
Phe Phe Gly Ala Val Ile Gly Thr Ile Ala Leu Gly Val Ala Thr Ala
1 5 10 15

<210> 16
<211> 16
<212> PRT
<213> respiratory syncytial virus

<400> 16
Phe Leu Gly Phe Leu Leu Gly Val Gly Ser Ala Ile Ala Ser Gly Val
1 5 10 15

<210> 17
<211> 16
<212> PRT
<213> Human immunodeficiency virus

<400> 17
Gly Val Phe Val Leu Gly Phe Leu Gly Phe Leu Ala Thr Ala Gly Ser
1 5 10 15

<210> 18
<211> 16
<212> PRT
<213> Ebola virus

<400> 18
Gly Ala Ala Ile Gly Leu Ala Trp Ile Pro Tyr Phe Gly Pro Ala Ala
1 5 10 15

<210> 19
<211> 56
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Exemplary zinc
finger motif

<400> 19
Ala Cys Thr Cys Pro Tyr Cys Lys Asp Ser Glu Gly Arg Gly Ser Gly
1 5 10 15

Asp Pro Gly Lys Lys Lys Gln His Ile Cys His Ile Gln Gly Cys Gly
20 25 30

Lys Val Tyr Gly Lys Thr Ser His Leu Arg Ala His Leu Arg Trp His
35 40 45

Thr Gly Glu Arg Pro Phe Met Cys
50 55

<210> 20
<211> 54
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Exemplary zinc
finger motif

```
<400> 20
Ala Cys Thr Cys Pro Asn Cys Lys Asp Gly Glu Lys Arg Ser Gly Glu
  1               5                  10                  15

Gln Gly Lys Lys Lys His Val Cys His Ile Pro Asp Cys Gly Lys Thr
             20                  25                  30

Phe Arg Lys Thr Ser Leu Leu Arg Ala His Val Arg Leu His Thr Gly
        35                  40                  45

Glu Arg Pro Phe Val Cys
     50


<210> 21
<211> 55
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Exemplary zinc
      finger motif

<400> 21
Ala Cys Thr Cys Pro Asn Cys Lys Glu Gly Gly Gly Arg Gly Thr Asn
  1               5                  10                  15

Leu Gly Lys Lys Lys Gln His Ile Cys His Ile Pro Gly Cys Gly Lys
             20                  25                  30

Val Tyr Gly Lys Thr Ser His Leu Arg Ala His Leu Arg Trp His Ser
        35                  40                  45

Gly Glu Arg Pro Phe Val Cys
     50                  55


<210> 22
<211> 56
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Exemplary zinc
      finger motif

<400> 22
Ala Cys Ser Cys Pro Asn Cys Arg Glu Gly Glu Gly Arg Gly Ser Asn
  1               5                  10                  15

Glu Pro Gly Lys Lys Lys Gln His Ile Cys His Ile Glu Gly Cys Gly
             20                  25                  30

Lys Val Tyr Gly Lys Thr Ser His Leu Arg Ala His Leu Arg Trp His
        35                  40                  45

Thr Gly Glu Arg Pro Phe Ile Cys
     50                  55
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<210> 23
<211> 60
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Exemplary zinc
finger motif

<400> 23
Arg Cys Thr Cys Pro Asn Cys Thr Asn Glu Met Ser Gly Leu Pro Pro
1 5 10 15

Ile Val Gly Pro Asp Glu Arg Gly Arg Lys Gln His Ile Cys His Ile
20 25 30

Pro Gly Cys Glu Arg Leu Tyr Gly Lys Ala Ser His Leu Lys Thr His
35 40 45

Leu Arg Trp His Thr Gly Glu Arg Pro Phe Leu Cys
50 55 60

<210> 24
<211> 58
<212> PRT
<213> Unknown Organism

<220>
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finger motif

<400> 24
Thr Cys Asp Cys Pro Asn Cys Gln Glu Ala Glu Arg Leu Gly Pro Ala
1 5 10 15

Gly Val His Ile Arg Lys Lys Asn Ile His Ser Cys His Ile Pro Gly
20 25 30

Cys Gly Lys Val Tyr Gly Lys Thr Ser His Leu Lys Ala His Leu Arg
35 40 45

Trp His Thr Gly Glu Arg Pro Phe Val Cys
50 55

<210> 25
<211> 53
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Exemplary zinc
finger motif

<400> 25
Arg Cys Thr Cys Pro Asn Cys Lys Ala Ile Lys His Gly Asp Arg Gly
1 5 10 15

Ser Gln His Thr His Leu Cys Ser Val Pro Gly Cys Gly Lys Thr Tyr
20 25 30

Lys Lys Thr Ser His Leu Arg Ala His Leu Arg Lys His Thr Gly Asp
35 40 45

Arg Pro Phe Val Cys
50

<210> 26
<211> 56
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Exemplary zinc
finger motif

<400> 26
Pro Gln Ile Ser Leu Lys Lys Lys Ile Phe Phe Phe Ile Phe Ser Asn
1 5 10 15

Phe Arg Gly Asp Gly Lys Ser Arg Ile His Ile Cys His Leu Cys Asn
20 25 30

Lys Thr Tyr Gly Lys Thr Ser His Leu Arg Ala His Leu Arg Gly His
35 40 45

Ala Gly Asn Lys Pro Phe Ala Cys
50 55

<210> 27
<211> 31
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Exemplary
polypeptide

<400> 27
Trp Trp Glu Thr Trp Lys Pro Phe Gln Cys Arg Ile Cys Met Arg Asn
1 5 10 15

Phe Ser Thr Arg Gln Ala Arg Arg Asn His Arg Arg Arg His Arg
20 25 30

<210> 28
<211> 16
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Exemplary
polypeptide

<400> 28
Gly Lys Ile Asn Leu Lys Ala Leu Ala Ala Leu Ala Lys Lys Ile Leu
1 5 10 15

<210> 29
<211> 16
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Exemplary
polypeptide

<400> 29
Arg Val Ile Arg Val Trp Phe Gln Asn Lys Arg Cys Lys Asp Lys Lys
1 5 10 15

<210> 30
<211> 39
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Exemplary
polypeptide

<400> 30
Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Arg Lys
1 5 10 15

Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Gly Arg Lys Lys Arg Arg
20 25 30

Gln Arg Arg Arg Pro Pro Gln
35

<210> 31
<211> 22
<212> PRT
<213> Unknown Organism

<220>
<223> Description of Unknown Organism: Exemplary
polypeptide

<400> 31
Gly Glu Gln Ile Ala Gln Leu Ile Ala Gly Tyr Ile Asp Ile Ile Leu
1 5 10 15

Lys Lys Lys Lys Ser Lys
20

<210> 32
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Combined DNA/RNA Molecule: Synthetic
siRNA

<220>
<223> Description of Artificial Sequence: Synthetic
siRNA

<400> 32
cuacacaaau cagcgauuut t 21

<210> 33
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Combined DNA/RNA Molecule: Synthetic
siRNA

<220>
<223> Description of Unknown Organism: Synthetic
siRNA

<400> 33
aaaucgcuga uuuguguagt t 21

<210> 34
<211> 22
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
siRNA

<400> 34
gcaagcugac ccugaaguuc au 22

<210> 35
<211> 29
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic

peptide

<400> 35
Arg Arg Arg Gln Arg Arg Lys Arg Gly Gly Asp Ile Met Gly Glu Trp
1 5 10 15

Gly Asn Glu Ile Phe Gly Ala Ile Ala Gly Phe Leu Gly
20 25

<210> 36
<211> 14
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 36
Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Cys
1 5 10

<210> 37
<211> 28
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 37
Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro
1 5 10 15

Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln
20 25

<210> 38
<211> 29
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 38
Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro
1 5 10 15

Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Cys
20 25

<210> 39
<211> 29
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 39
Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Cys Ala Ala Val
1 5 10 15

Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro
20 25

<210> 40
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 40
Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Gln
1 5 10

<210> 41
<211> 29
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 41
Arg Arg Arg Gln Arg Arg Lys Arg Gly Gly Asp Ile Met Gly Glu Trp
1 5 10 15

Gly Asn Glu Ile Phe Gly Ala Ile Ala Gly Phe Leu Gly
20 25

<210> 42
<211> 25
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 42
Cys Tyr Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg Gly Tyr Gly Arg
1 5 10 15

Lys Lys Arg Arg Gln Arg Arg Arg Gly
20 25

<210> 43
<211> 13
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 43
Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln
1 5 10

<210> 44
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 44
Lys Leu Trp Lys Ala Trp Pro Lys Leu Trp Lys Lys Leu Trp Lys Pro
1 5 10 15

<210> 45
<211> 22
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 45
Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro
1 5 10 15

Arg Arg Arg Arg Arg Arg
20

<210> 46
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 46
Arg Leu Trp Arg Ala Leu Pro Arg Val Leu Arg Arg Leu Leu Arg Pro
1 5 10 15

<210> 47
<211> 28
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 47
Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro
1 5 10 15

Ser Gly Ala Ser Gly Leu Asp Lys Arg Asp Tyr Val
20 25

<210> 48
<211> 28
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 48
Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro
1 5 10 15

Ser Gly Ala Ser Gly Leu Asp Lys Arg Asp Tyr Val
20 25

<210> 49
<211> 29
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 49
Ser Gly Ala Ser Gly Leu Asp Lys Arg Asp Tyr Val Ala Ala Val Ala
1 5 10 15

Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro
20 25

<210> 50
<211> 33
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 50
Leu Leu Glu Thr Leu Leu Lys Pro Phe Gln Cys Arg Ile Cys Met Arg
1 5 10 15

Asn Phe Ser Thr Arg Gln Ala Arg Arg Asn His Arg Arg Arg His Arg
20 25 30

Arg

<210> 51
<211> 27
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 51
Ala Ala Val Ala Cys Arg Ile Cys Met Arg Asn Phe Ser Thr Arg Gln
1 5 10 15

Ala Arg Arg Asn His Arg Arg Arg His Arg Arg
20 25

<210> 52
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 52
Arg Gln Ile Lys Ile Trp Phe Gln Asn Arg Arg Met Lys Trp Lys Lys
1 5 10 15

<210> 53
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      peptide

<400> 53
Arg Gln Ile Lys Ile Trp Phe Gln Asn Arg Arg Met Lys Trp Lys Lys
  1   5   10   15

<210> 54
<211> 35
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      peptide

<400> 54
Arg Gln Ile Lys Ile Trp Phe Gln Asn Arg Arg Met Lys Trp Lys Lys
  1   5   10   15

Asp Ile Met Gly Glu Trp Gly Asn Glu Ile Phe Gly Ala Ile Ala Gly
             20   25   30

Phe Leu Gly
         35

<210> 55
<211> 37
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      peptide

<400> 55
Ser Gly Arg Gly Lys Gln Gly Gly Lys Ala Arg Ala Lys Ala Lys Thr
  1   5   10   15

Arg Ser Ser Arg Ala Gly Leu Gln Phe Pro Val Gly Arg Val His Arg
             20   25   30

Leu Leu Arg Lys Gly
         35

<210> 56
<211> 38
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      peptide

<400> 56
Ser Gly Arg Gly Lys Gln Gly Gly Lys Ala Arg Ala Lys Ala Lys Thr
1 5 10 15

Arg Ser Ser Arg Ala Gly Leu Gln Phe Pro Val Gly Arg Val His Arg
20 25 30

Leu Leu Arg Lys Gly Cys
35

<210> 57
<211> 23
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 57
Lys Gly Ser Lys Lys Ala Val Thr Lys Ala Gln Lys Lys Asp Gly Lys
1 5 10 15

Lys Arg Lys Arg Ser Arg Lys
20

<210> 58
<211> 25
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 58
Lys Lys Asp Gly Lys Lys Arg Lys Arg Ser Arg Lys Glu Ser Tyr Ser
1 5 10 15

Val Tyr Val Tyr Lys Val Leu Lys Gln
20 25

<210> 59
<211> 36
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 59
Lys Gly Ser Lys Lys Ala Val Thr Lys Ala Gln Lys Lys Asp Gly Lys
1 5 10 15

Lys Arg Lys Arg Ser Arg Lys Glu Ser Tyr Ser Val Tyr Val Tyr Lys
20 25 30

Val Leu Lys Gln
35

<210> 60
<211> 27
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 60
Gly Trp Thr Leu Asn Ser Ala Gly Tyr Leu Leu Gly Lys Ile Asn Leu
1 5 10 15

Lys Ala Leu Ala Ala Leu Ala Lys Lys Ile Leu
20 25

<210> 61
<211> 18
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 61
Lys Leu Ala Leu Lys Leu Ala Leu Lys Ala Leu Lys Ala Ala Leu Lys
1 5 10 15

Leu Ala

<210> 62
<211> 21
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 62
Lys Glu Thr Trp Trp Glu Thr Trp Trp Thr Glu Trp Ser Gln Pro Lys
1 5 10 15

Lys Lys Arg Lys Val
20

```
<210> 63
<211> 28
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      peptide

<400> 63
Lys Glu Thr Trp Trp Glu Thr Trp Trp Thr Glu Trp Ser Gln Pro Gly
  1               5                  10                  15

Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln
             20                  25


<210> 64
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      peptide

<400> 64
Arg Arg Arg Arg Arg Arg Arg
  1               5


<210> 65
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      peptide

<400> 65
Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln
  1               5                  10


<210> 66
<211> 20
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      peptide

<400> 66
Arg Arg Arg Gln Arg Arg Lys Arg Gly Gly Gln Gln Gln Gln Gln Gln
  1               5                  10                  15
```

Gln Gln Gln Gln
20

<210> 67
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 67
Arg Val Ile Arg Trp Phe Gln Asn Lys Arg Cys Lys Asp Lys Lys
1 5 10 15

<210> 68
<211> 18
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 68
Leu Gly Leu Leu Leu Arg His Leu Arg His His Ser Asn Leu Leu Ala
1 5 10 15

Asn Ile

<210> 69
<211> 18
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 69
Gly Gln Met Ser Glu Ile Glu Ala Lys Val Arg Thr Val Lys Leu Ala
1 5 10 15

Arg Ser

<210> 70
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      peptide

<400> 70
```
Lys Leu Trp Ser Ala Trp Pro Ser Leu Trp Ser Ser Leu Trp Lys Pro
 1               5                  10                  15
```

<210> 71
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      peptide

<400> 71
```
Lys Lys Lys Lys Lys Lys Lys Lys Lys
 1               5
```

<210> 72
<211> 21
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      peptide

<400> 72
```
Ala Ala Arg Leu His Arg Phe Lys Asn Lys Gly Lys Asp Ser Thr Glu
 1               5                  10                  15

Met Arg Arg Arg Arg
             20
```

<210> 73
<211> 22
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
      peptide

<400> 73
```
Gly Leu Gly Ser Leu Leu Lys Lys Ala Gly Lys Lys Leu Lys Gln Pro
 1               5                  10                  15

Lys Ser Lys Arg Lys Val
             20
```

<210> 74
<211> 14
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<220>
<221> MOD_RES
<222> (1)
<223> DiMethylTyrosine

<400> 74
Xaa Arg Phe Lys Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln
1 5 10

<210> 75
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 75
Trp Arg Phe Lys
1

<210> 76
<211> 14
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 76
Trp Arg Phe Lys Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln
1 5 10

<210> 77
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 77

Tyr Arg Phe Lys
1

<210> 78
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 78
Tyr Arg Phe Lys Tyr Arg Phe Lys Tyr Arg Phe Lys
1 5 10

<210> 79
<211> 10
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 79
Trp Arg Phe Lys Lys Ser Lys Arg Lys Val
1 5 10

<210> 80
<211> 20
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 80
Trp Arg Phe Lys Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala
1 5 10 15

Leu Leu Ala Pro
20

<210> 81
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<220>
<221> MOD_RES
<222> (1)
<223> DiMethylTyrosine

<400> 81
Xaa Arg Phe Lys
1

<210> 82
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 82
Tyr Arg Phe Lys
1

<210> 83
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<220>
<221> MOD_RES
<222> (1)
<223> DiMethylTyrosine

<400> 83
Xaa Arg Phe Lys
1

<210> 84
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 84
Trp Arg Phe Lys
1

<210> 85
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<220>
<221> MOD_RES
<222> (1)
<223> DiMethylTyrosine

<400> 85
Xaa Tyr Arg Trp Lys
1 5

<210> 86
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<220>
<221> MOD_RES
<222> (4)
<223> DiMethylTyrosine

<400> 86
Lys Phe Arg Xaa
1

<210> 87
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 87
Trp Arg Phe Lys Trp Arg Phe Lys
1 5

<210> 88
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 88
Trp Arg Phe Lys Trp Arg Phe Lys Trp Arg Phe Lys
1 5 10

<210> 89
<211> 28
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 89
Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Ala Ala Val Ala
1 5 10 15

Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro
20 25

<210> 90
<211> 36
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 90
Lys Gly Ser Lys Lys Ala Val Thr Lys Ala Gln Lys Lys Asp Gly Lys
1 5 10 15

Lys Arg Lys Arg Ser Arg Lys Glu Ser Tyr Ser Val Tyr Val Tyr Lys
20 25 30

Val Leu Lys Gln
35

<210> 91
<211> 36
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 91
Arg Gly Ser Arg Arg Ala Val Thr Arg Ala Gln Arg Arg Asp Gly Arg
1 5 10 15

Arg Arg Arg Arg Ser Arg Arg Glu Ser Tyr Ser Val Tyr Val Tyr Arg
20 25 30

Val Leu Arg Gln
35

<210> 92
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 92
Arg Val Ile Arg Trp Phe Gln Asn Lys Arg Ser Lys Asp Lys Lys
1 5 10 15

<210> 93
<211> 27
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 93
Gly Trp Thr Leu Asn Ser Ala Gly Tyr Leu Leu Gly Lys Ile Asn Leu
1 5 10 15

Lys Ala Leu Ala Ala Leu Ala Lys Lys Ile Leu
20 25

<210> 94
<211> 28
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 94
Ala Ala Val Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro
1 5 10 15

Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln
20 25

<210> 95
<211> 21
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 95
Lys Glu Thr Trp Trp Glu Thr Trp Trp Thr Glu Trp Ser Gln Pro Lys
1 5 10 15

Lys Lys Arg Lys Val
20

<210> 96
<211> 28
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 96
Gly Ala Leu Phe Leu Gly Phe Leu Gly Ala Ala Gly Ser Thr Met Gly
1 5 10 15

Ala Trp Ser Gln Pro Lys Ser Lys Arg Lys Val Cys
20 25

<210> 97
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 97
Arg Lys Glu Ser Tyr Ser Val Tyr Val Tyr Lys Val Leu Lys Gln
1 5 10 15

<210> 98
<211> 36
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 98
Lys Gly Ser Lys Lys Ala Val Thr Lys Ala Gln Lys Lys Asp Gly Lys
1 5 10 15

Lys Arg Lys Arg Ser Arg Lys Glu Ser Tyr Ser Val Tyr Val Tyr Lys
20 25 30

Val Leu Lys Gln
35

<210> 99
<211> 33
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 99
Lys Lys Ala Val Thr Lys Ala Gln Lys Lys Asp Gly Lys Lys Arg Lys
1 5 10 15

Arg Ser Arg Lys Glu Ser Tyr Ser Val Tyr Val Tyr Lys Val Leu Lys
20 25 30

Gln

<210> 100
<211> 30
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 100
Val Thr Lys Ala Gln Lys Lys Asp Gly Lys Lys Arg Lys Arg Ser Arg
1 5 10 15

Lys Glu Ser Tyr Ser Val Tyr Val Tyr Lys Val Leu Lys Gln
20 25 30

<210> 101
<211> 27
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 101
Ala Gln Lys Lys Asp Gly Lys Lys Arg Lys Arg Ser Arg Lys Glu Ser
1 5 10 15

Tyr Ser Val Tyr Val Tyr Lys Val Leu Lys Gln
20 25

<210> 102
<211> 29
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 102
Arg Arg Arg Gln Arg Arg Lys Arg Gly Gly Asp Ile Met Gly Glu Trp
1 5 10 15

Gly Asn Glu Ile Phe Gly Ala Ile Ala Gly Phe Leu Gly
20 25

<210> 103
<211> 36
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 103
Lys Gly Ser Lys Lys Ala Val Thr Lys Ala Gln Lys Lys Asp Gly Lys
1 5 10 15

Lys Arg Lys Arg Ser Arg Lys Glu Ser Tyr Ser Val Tyr Val Tyr Lys
20 25 30

Val Leu Lys Gln
35

<210> 104
<211> 21
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 104
Lys Glu Thr Trp Trp Glu Thr Trp Trp Thr Glu Trp Ser Gln Pro Lys
1 5 10 15

Lys Lys Arg Lys Val
20

<210> 105
<211> 29
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 105
Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Gln Cys Ala Ala Val
1 5 10 15

Ala Leu Leu Pro Ala Val Leu Leu Ala Leu Leu Ala Pro
20 25

<210> 106
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 106
Trp Arg Phe Lys Cys
1 5

<210> 107
<211> 18
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 107
Lys Leu Ala Leu Lys Leu Ala Leu Lys Ala Leu Lys Ala Ala Leu Lys
1 5 10 15

Leu Ala

<210> 108
<211> 27
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 108
Gly Trp Thr Leu Asn Ser Ala Gly Tyr Leu Leu Gly Lys Ile Asn Leu
1 5 10 15

Lys Ala Leu Ala Ala Leu Ala Lys Lys Ile Leu
20 25

<210> 109
<211> 19
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
siRNA

<400> 109
gcguggagcu gagagauaa 19

<210> 110
<211> 19
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
siRNA

<400> 110
gccuguagcc cauguugua 19

<210> 111
<211> 19
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
siRNA

<400> 111
gguaugagcc caucuaucu 19

<210> 112
<211> 19
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic

siRNA

<400> 112
ccagggaccu cucucuaau 19

<210> 113
<211> 19
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
siRNA

<400> 113
gcccgacuau cucgacuuu 19

<210> 114
<211> 19
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
siRNA

<400> 114
ugacaagccu guagcccau 19

<210> 115
<211> 19
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
siRNA

<400> 115
ggucuacuuu gggaucauu 19

<210> 116
<211> 19
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
siRNA

<400> 116
cccagggacc ucucucuaa 19

<210> 117
<211> 23
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
siRNA

<400> 117
aaucggcccg acuaucucga cuu 23

<210> 118
<211> 21
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
siRNA

<400> 118
aauggcgugg agcugagaga u 21

<210> 119
<211> 21
<212> RNA
<213> Artificial Sequence

<220>
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siRNA

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<210> 120
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<220>
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<210> 121
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aaucucgacu uugccgaguc u 21

<210> 122
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<220>
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siRNA

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aagggugacc gacucagcgc u 21

<210> 123
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<210> 124
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aagcuccagu ggcugaaccg c 21

<210> 126
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aagucagauc aucuucucga a 21

<210> 127
<211> 21
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<220>
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siRNA

<400> 127
aagggaccuc ucucuaauca g 21

<210> 128
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<213> Artificial Sequence

<220>
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siRNA

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ccucagccuc uucuccuucc uga 23

<210> 129
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siRNA

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aauccucagc cucuucuccu u 21

<210> 130
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siRNA

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cugauuaagu ugucuaaaca a 21

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<210> 133
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cuugugauua uuuauuauuu a 21

<210> 134
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aagccuguag cccauguugu a 21

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<400> 135
uagggucgga acccaagcuu a 21

<210> 136
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<400> 136
cugaaagcau gauccggga 19

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<220>
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siRNA

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aggcggugcu uguuccuca 19

<210> 138
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agggaccucu cucuaauca 19

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<210> 141
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<400> 141
gccuguagcc cauguugua 19

<210> 142
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<220>
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uagcccaugu uguagcaaa 19

<210> 143
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<400> 143
ccaaugcccu ccuggccaa 19

<210> 144
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ccaauggcgu ggagcugag 19

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ggcguggagc ugagagaua 19

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gcguggagcu gagagauaa 19

<210> 147
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<210> 148
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ccuccucucu gccaucaag 19

<210> 149
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gguaugagcc caucuaucu 19

<210> 150
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siRNA

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gcuggagaag ggugaccga 19

<210> 151
<211> 19
<212> RNA
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siRNA

<400> 151
gagaagggug accgacuca 19

<210> 152
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siRNA

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gcccgacuau cucgacuuu 19

<210> 153
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<400> 153
gcaggucuac uuugggauc 19

<210> 154
<211> 19
<212> RNA
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siRNA

<400> 154
ggucuacuuu gggaucauu 19

<210> 155
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<212> RNA
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<400> 155
ugggaucauu gcccuguga 19

<210> 156
<211> 19
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<220>
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siRNA

<400> 156
ggucggaacc caagcuuag 19

<210> 157
<211> 19
<212> RNA
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siRNA

<400> 157
ccagaaugcu gcaggacuu 19

<210> 158
<211> 19
<212> RNA
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<220>
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siRNA

<400> 158
gagaagaccu caccuagaa 19

<210> 159
<211> 19
<212> RNA
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<220>
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siRNA

<400> 159
gaagaccuca ccuagaaau 19

<210> 160
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<212> RNA
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<220>
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siRNA

<400> 160
ccagauguuu ccagacuuc 19

<210> 161
<211> 19
<212> RNA
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<220>
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siRNA

<400> 161
cuauuuaugu uugcacuug 19

<210> 162
<211> 19
<212> RNA
<213> Artificial Sequence

<220>
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siRNA

<400> 162
ucuaaacaau gcugauuug 19

<210> 163
<211> 18
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
siRNA

<400> 163
gaccaacugu cacucauu 18

<210> 164
<211> 125
<212> PRT
<213> Homo sapiens

<400> 164
Met Pro Glu Pro Ala Lys Ser Ala Pro Ala Pro Lys Lys Gly Ser Lys
1 5 10 15

Lys Ala Val Thr Lys Ala Gln Lys Lys Asp Ser Lys Lys Arg Lys Arg
20 25 30

Ser Arg Lys Glu Ser Tyr Ser Val Tyr Val Tyr Lys Val Leu Lys Val
35 40 45

His Pro Asp Thr Gly Ile Ser Ser Lys Ala Met Gly Ile Met Asn Ser
50 55 60

Phe Val Asn Asp Ile Phe Glu Arg Ile Ala Gly Glu Ala Ser Arg Leu
65 70 75 80

Ala His Tyr Asn Lys Arg Ser Thr Ile Thr Ser Arg Glu Ile Gln Thr
85 90 95

Ala Val Arg Leu Leu Leu Pro Gly Glu Leu Ala Lys His Ala Val Ser
100 105 110

Glu Gly Thr Lys Ala Val Thr Lys Tyr Thr Ser Ser Lys
115 120 125

<210> 165
<211> 24
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 165
Lys Asp Gly Lys Lys Arg Lys Arg Ser Arg Lys Glu Ser Tyr Ser Val
1 5 10 15

Tyr Val Tyr Lys Val Leu Lys Gln
20

<210> 166
<211> 21
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 166
Lys Lys Arg Lys Arg Ser Arg Lys Glu Ser Tyr Ser Val Tyr Val Tyr
1 5 10 15

Lys Val Leu Lys Gln
20

<210> 167
<211> 18
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 167
Lys Arg Ser Arg Lys Glu Ser Tyr Ser Val Tyr Val Tyr Lys Val Leu
1 5 10 15

Lys Gln

<210> 168
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 168
Arg Lys Glu Ser Tyr Ser Val Tyr Val Tyr Lys Val Leu Lys Gln
1 5 10 15

<210> 169
<211> 12
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 169
Ser Tyr Ser Val Tyr Val Tyr Lys Val Leu Lys Gln
1 5 10

<210> 170
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 170
Val Tyr Val Tyr Lys Val Leu Lys Gln
1 5

<210> 171
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 171
Tyr Lys Val Leu Lys Gln
1 5

<210> 172
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 172
Lys Val Leu Lys Gln
1 5

<210> 173
<211> 36
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 173
Lys Gly Ser Lys Lys Ala Val Thr Lys Ala Gln Lys Lys Asp Gly Lys
1 5 10 15

Lys Arg Lys Arg Ser Arg Lys Glu Ser Tyr Trp Val Tyr Val Tyr Lys
20 25 30

Val Leu Lys Gln
35

<210> 174
<211> 36
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 174
Lys Gly Ser Lys Lys Ala Val Thr Lys Ala Gln Lys Lys Asp Gly Lys
1 5 10 15

Lys Arg Lys Arg Ser Arg Lys Trp Ser Tyr Ser Val Tyr Val Tyr Lys
20 25 30

Val Leu Lys Gln
35

<210> 175
<211> 36
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 175
Lys Gly Ser Lys Lys Ala Val Thr Lys Ala Gln Lys Lys Asp Gly Lys
1 5 10 15

Lys Arg Lys Arg Ser Arg Lys Phe Ser Tyr Ser Val Tyr Val Tyr Lys
20 25 30

Val Leu Lys Gln
35

<210> 176
<211> 36
<212> PRT
<213> Artificial Sequence

<220>
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peptide

<400> 176
Lys Gly Ser Phe Lys Ala Val Thr Lys Ala Gln Lys Lys Asp Gly Lys
1 5 10 15

Lys Arg Lys Arg Ser Phe Lys Phe Ser Tyr Ser Val Tyr Val Tyr Lys
20 25 30

Val Leu Lys Gln
35

<210> 177
<211> 35
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 177
Lys Gly Ser Phe Lys Ala Val Thr Lys Ala Gln Lys Lys Phe Gly Lys
1 5 10 15

Lys Arg Lys Arg Ser Arg Lys Ser Phe Ser Val Tyr Val Tyr Lys Val
20 25 30

Leu Lys Gln
35

<210> 178
<211> 25
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 178
Arg Ser Val Cys Arg Gln Ile Lys Ile Cys Arg Arg Arg Gly Gly Cys
1 5 10 15

Tyr Tyr Lys Cys Thr Asn Arg Pro Tyr
20 25

<210> 179
<211> 33
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 179
Gly Phe Phe Ala Leu Ile Pro Lys Ile Ile Ser Ser Pro Leu Phe Lys
1 5 10 15

Thr Leu Leu Ser Ala Val Gly Ser Ala Leu Ser Ser Ser Gly Asp Gln
20 25 30

Glu

<210> 180
<211> 24
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 180
Gly Thr Ala Met Arg Ile Leu Gly Gly Val Ile Pro Arg Lys Lys Arg
1 5 10 15

Arg Gln Arg Arg Arg Pro Pro Gln
20

<210> 181
<211> 25
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 181
Lys Lys Lys Lys Lys Arg Phe Ser Phe Lys Lys Ser Phe Lys Leu Ser
1 5 10 15

Gly Phe Ser Phe Lys Lys Asn Lys Lys
20 25

<210> 182
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 182
Arg Gln Ile Lys Ile Trp Phe Gln Asn Arg Arg Met Lys Trp Lys Lys
1 5 10 15

<210> 183
<211> 16
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 183
Arg Gln Ile Arg Ile Trp Phe Gln Asn Arg Arg Met Arg Trp Arg Arg
1 5 10 15

<210> 184
<211> 41
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
peptide

<400> 184
Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro Val Ala Tyr Ile Ser
1 5 10 15

Arg Gly Gly Val Ser Thr Tyr Tyr Ser Asp Thr Val Lys Gly Arg Phe
20 25 30

Thr Arg Gln Lys Tyr Asn Lys Arg Ala
35 40

<210> 185
<211> 30
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 185
Leu Gly Leu Leu Leu Arg His Leu Arg His His Ser Asn Leu Leu Ala
1 5 10 15

Asn Ile Pro Arg Lys Lys Arg Arg Gln Arg Arg Arg Pro Pro
20 25 30

<210> 186
<211> 21
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic peptide

<400> 186
Lys Glu Thr Trp Trp Glu Thr Trp Trp Thr Glu Trp Ser Gln Pro Lys
1 5 10 15

Lys Lys Arg Lys Val
20

<210> 187
<211> 19
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic siRNA

<400> 187
uagcccaugu uguagcaaa 19

<210> 188
<211> 23
<212> PRT
<213> Artificial Sequence

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<220>
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      formula peptide

<220>
<221> MOD_RES
<222> (2)..(5)
<223> this region may encompas 2 or 4 variable residues

<220>
<221> MOD_RES
<222> (7)..(18)
<223> variable residue

<220>
<221> MOD_RES
<222> (20)..(22)
<223> variable residue

<400> 188
Cys Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
  1               5                  10                  15

Xaa Xaa His Xaa Xaa Xaa His
             20
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